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UNIVERSITY OF ALBERTA

NUCLEIC ACID HYBRIDIZATION STUDIES IN EPSTEIN-BARR VIRUS INFECTIONS

By

FRANCISCO JAVIER DIAZ-MITOMA

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES (VIROLOGY)

DEPARTMENT OF MEDICAL MICROBIOLOGY
AND INFECTIOUS DISEASES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, "Nucleic Acid Hybridization Studies in Epstein-Barr Virus Infections," submitted by Francisco Javier Diaz-Mitoma in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Virology.

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To Alejandra, Rodrigo, and Francisco,
who are a continuous source of love and happiness.

To my parents, Francisco, Amelia, Hidesaku, and Quita,
who taught me the basics of life.

ABSTRACT

The EBV-specific serology and the lymphocyte transformation assay are not ideal methods with which to study the role of EBV in clinical problems such as chronic headaches, HIV infection, and lymphoproliferative disorders in the immunocompromised host. Nucleic acid hybridization techniques were developed and applied to some of these syndromes in which EBV has been hypothesized to play a role. The use of dot hybridization to detect EBV DNA extracted from oropharyngeal cells was validated by comparing it to the lymphocyte transformation assay. The sensitivity and specificity of the dot hybridization were 90% and 98%, respectively. This assay was used to investigate whether there was an association between new daily persistent headaches (NDPH) and EBV infection. The dot hybridization was positive in 20 (60%) of 32 headache patients and 4 (12%) of 32 control subjects ($p < 0.001$). The precise role of the virus in headache syndromes needs further study.

In order to study the effects of EBV infection in the progression of HIV-induced disease, a quantitative assay to measure the levels of EBV in oropharyngeal cells was developed. The predictive value of several markers of progressive HIV infection, such as T4/T8, p24 core antigenemia, CMV serology, EBV serology, and levels of EBV DNA were examined. A high level of EBV excretion was the best single predictor of progression of HIV infection ($p < 0.001$). These results suggest EBV may be of significance in the natural history of HIV infection.

During latency, episomal forms of the virus are replicated by cellular enzymes, and antiviral agents have no effect on EBV replication. In comparison, viral

enzymes, which may be inhibited by antiviral agents, replicate the linear forms of the viral genome. The intracellular configuration of the EBV genome was examined in oropharyngeal cells. A preponderance of linear forms was found *in vivo*. It is predicted that high levels of EBV excretion, as detected by dot hybridization, may be inhibited by antiviral therapy. Finally, the levels of EBV excretion and the prevalence of EBV infections by type A and type B variants were studied in patients with lymphoproliferative disorder (LPD) after organ transplantation. A high level of EBV excretion was a good predictor for the development of LPD; only infections caused by type A variants were found in patients with LPD.

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ABBREVIATIONS AND DEFINITIONS

ACV	Acycloguanosine [9-(2-hydroxyethoxymethyl)-guanine]
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ARC	AIDS-related complex
AZT	Azidothymidine
B cell/lymphocyte	Bone marrow-derived lymphocyte
bp	Base pairs
CBC	Cell blood count
CMV	Cytomegalovirus
CNS	Central nervous system
cpm	Counts per minute
C3dR	Receptor for the 3d component of complement
DNA	Deoxyribonucleic acid
EA	Early antigen
EA(D)	Early antigen (diffuse)
EA(R)	Early antigen (restricted)
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	Edetic acid
ELISA	Enzyme-linked immunosorbent assay
HHV-6	Human herpesvirus 6
HIV-1	Human immunodeficiency virus type 1
HSV	Herpes simplex virus
HVS	Herpes virus saimiri
IFN	Interferon
Ig	Immunoglobulin
IL2	Interleukin 2
IM	Infectious mononucleosis
IR	Internal repeat
IV	Intravenous

kb(p)	Kilo base (pairs)
kDa	KiloDaltons
LAT	Latency-associated transcript
LPD	Lymphoproliferative disorder
LYDMA	Lymphocyte-detected membrane antigen
MA	Membrane antigen
mRNA	Messenger ribonucleic acid
NDPH	New daily persistent headaches
PCR	Polymerase chain reaction
(^{32}P)-dCTP	Deoxy-cytidine triphosphate labeled with radioactive phosphate
PRV	Pseudorabies virus
SDS	Sodium dodecyl sulphate
SSC	Sodium citrate
T cell/lymphocyte	Thymus-dependent lymphocyte
Taq	Thermophilus acquaticus
TPA	12-0-tetradecanoyl-phorbol-13-acetate
TR	Terminal repeat
ts	Temperature-sensitive
U	Unique region
VCA	Viral capsid antigen
VZV	Varicella-zoster virus
XLP	X-linked lymphoproliferative syndrome

CHAPTER I

INTRODUCTION

THE HERPESVIRUSES

Introduction to the Herpesviruses

The family Herpesviridae is characterized by a group of viruses containing a double-stranded linear DNA, a capsid with icosahedral symmetry containing 162 capsomeres, and an envelope derived from the nuclear membrane of the host cell. More than 80 herpesviruses have been isolated from a wide variety of animal species (Roizman and Batterson, 1985). Six herpesviruses have been isolated from humans [herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and the newly identified human herpesvirus 6 (HHV-6)].

General Features of the Herpesviruses

All human herpesviruses share some biologic properties, including their ability to cause latent infections and to infect epithelial cells and cells of the immune system. In contrast, major differences exist in their genome structure, specific features of their reproductive cycle, and their effects on host cells.

Herpesviruses are an important cause of morbidity and mortality in the human population. The diseases associated with human herpesviruses are summarized below.

Diseases Associated with Human Herpesviruses

Herpes Simplex Virus:

During primary infection with HSV-1 or HSV-2, the viral genome replicates in the skin or epithelium, and virions then ascend by retrograde axonal transport in sensory nerve cells. Infection of neuronal tissue results in either viral replication, causing death of the host cell, or establishment of a latent infection and neuronal survival. In latent infections, the viral genome may be reactivated, and virions descend by anterograde axonal transport to infect the epithelium, resulting in symptomatic or asymptomatic virus shedding. HSV-1 causes the common cold sore and is the most common viral cause of blindness and sporadic encephalitis. It may also cause aggressive infection in immunosuppressed patients. HSV-2 causes genital herpes and may also cause fulminant disease when transmitted to neonates.

Varicella Zoster Virus:

VZV is the cause of chickenpox and herpeszoster (shingles). In the immunocompromised host it may cause disseminated and occasionally fatal infections. The pathogenesis of VZV in chickenpox involves a primary asymptomatic infection of the oropharyngeal or conjunctival epithelium followed by a viremia, viral replication in reticuloendothelial cells, and a secondary viremia followed by infection of the skin and mucous membranes. Shingles is the counterpart of the common cold sore in varicella zoster virus infections. The reactivation of a latent infection in sensory ganglia results in active replication of the virus, anterograde axonal transport, liberation of virions from the axons, and, finally, dermatomal skin lesions.

Epstein-Barr Virus:

EBV causes heterophil antibody-positive acute infectious mononucleosis in young adults. It has also been associated with lymphoproliferative disorders in organ transplant recipients and HIV-seropositive individuals. EBV may also have an etiologic role in nasopharyngeal carcinoma and African Burkitt's lymphoma.

The pathogenesis of acute infectious mononucleosis involves the replication of the virus in the oropharyngeal epithelium, followed by infection of B lymphocytes and an intense humoral and cellular immune response that results in the classical manifestations of infectious mononucleosis. These include lymphadenopathy, fever, and sore throat. Active EBV replication, characterized by virus shedding in saliva, may be prolonged for weeks or months after infectious mononucleosis and during episodes of immunosuppression.

Cytomegalovirus:

The pathogenesis of CMV infection involves replication of the viral genome in epithelial cells of the oropharynx with shedding of the virus in saliva, followed by a viremia. CMV has a predilection for leukocytes and particularly for tubular epithelial cells of the kidney, where a permissive infection results in prolonged shedding of the virus in the urine.

CMV infection may result in an infectious mononucleosis-like syndrome with atypical lymphocytes, but lacking heterophil antibodies. Most CMV infections are mild or asymptomatic. Nevertheless, severe and lethal CMV infections are seen, primarily in immunocompromised individuals. Transplacental CMV infections causing congenital malformations may occur during pregnancy.

Human Herpes Virus 6:

HHV-6 was first isolated in 1986 from patients with lymphoproliferative disorders (Salahuddin *et al.*, 1986). Although the pathogenesis of HHV-6 infections is not clear, it is now known that HHV-6 not only infects B lymphocytes, but also replicates preferentially in T cell lymphocytes (Lopez *et al.*, 1988; Agut *et al.*, 1988). The prevalence of HHV-6 seropositivity varies geographically. For instance, in the UK 52% of healthy blood donors are seropositive (Briggs *et al.*, 1988), and in Austria only 8% of the blood donors and 16% of HIV-seropositive individuals are HHV-6-seropositive (Huemer *et al.*, 1989). Initial infection usually occurs during childhood, causing exanthem subitum or roseola infantum (Yamanishi *et al.*, 1988). HHV-6 shares with other herpesviruses the ability to remain latent after infection and may be activated during episodes of immunosuppression.

Latency of the Herpesviruses

Latency may be defined as the capacity of the virus to remain intracellularly with only partial expression of its genome. Infection with herpesviruses is usually followed by latency. The molecular mechanisms operative during establishment and maintenance of latency and during reactivation from the latent state are being actively studied. In mouse model systems, DNA extracted from the herpes simplex virus in the latent state differs from DNA extracted during the acute infection or from DNA extracted from purified virions. The genomes from the latent virus lack detectable free ends, suggesting that they are integrated into multiple sites of the host genome or are present in an extrachromosomal form

(Rock and Fraser, 1985) such as multimeric linear, circular molecules, or monomeric circles. A more recent study of murine brains latently infected with HSV-1 suggests that the most likely physical form of the latent virus is circular (Mellerick and Fraser, 1987). Specific viral genes, as well as characteristics of the infected cells, probably determine whether a viral infection will result in latency or permissive viral replication. Viral gene expression in tissues latently infected with HSV-1 is restricted to the latency-associated transcripts (LATs) derived from the long terminal repeat (TR) of the genome. Two LATs, of 2.0 and 1.3 kb, are transcribed from the TR during latency. The 3' portions of the LATs are complementary to the 3' ends of the mRNA encoding the immediate-early protein Vmw110 (or ICP0), suggesting that the LATs might act as anti-sense transcripts which inhibit the expression of Vmw110. Vmw110 is involved in the control of the initial viral replicative process (Wechsler *et al.*, 1989). However, a recent study by Sedarati *et al.* (1989) suggests that the LATs are not essential in order to maintain latent infection. The number of viral genomes per infected ganglion was no different in neuronal cells expressing LATs than in those not expressing them. Further studies are necessary to resolve the current controversy regarding the molecular basis of latency.

Molecular Biology of the Herpesviruses

Anatomy of the Herpesvirus DNAs:

The DNA extracted from members of the family Herpesviridae are linear, double-stranded molecules ranging in molecular weights from 80 to 150 million. Herpesvirus DNAs contain terminally and internally repeated nucleotide

sequences. Because of variability in the number of reiterations, the molecular weights within species may vary by as much as 20 kilobase pairs. Herpesvirus DNAs may be subdivided into three classes according to their sequence arrangements (Hones and Watson, 1977). These are illustrated in Figure I-1. The DNA of Class I is characterized by the presence of direct repeats. No inverted repeats have been demonstrated in this class of DNA molecules. The DNA of Class II contains one set of inverted repeats bracketing a unique sequence of nucleotides. The DNA of Class III contains two sets of repeats, each bracketing a unique sequence of DNA. Although the genome of EBV belongs to Class 1 DNA, it differs from other members within this class series in containing four series of tandem reiterations at internal sites within the molecule (Hayward *et al.*, 1980).

DNA Homology Among the Herpesviruses:

Few regions of DNA homology can be demonstrated between human herpesvirus DNAs of different genera (Ben-Porat *et al.*, 1983). In contrast, viruses of the same genus show regions of DNA homology. For example, members of the simplex virus genus HSV-1 and HSV-2 have regions of genomic DNA homology (Kieff *et al.*, 1972). In addition, the viruses in the genus *Lymphocryptovirus*, such as EBV and the chimpanzee herpesviruses, also contain regions of DNA homology (Gerber *et al.*, 1976).

DNA Heterogeneity Within the Same Herpesvirus Species:

Two types of variability are known to occur in herpesvirus DNA. The first type of variability is found in the number of reiterations in the telomeric and internal tandemly repeated sequences of EBV and HSV (Bornkamm *et al.*, 1980;

Stow and McMonagle, 1983). The second type of variability is found in the number and location of restriction-enzyme cleavage sites. Strains of HSV-1, HSV-2, CMV, VZV, and HHV-6 that are epidemiologically different demonstrate diversity in their restriction-enzyme pattern. The observation that herpesviruses may be differentiated on the basis of the polymorphisms in DNA fragments generated by restriction endonuclease digestion has provided an important tool with which to trace the transmission and behavior of herpesviruses (reviewed in Roizman and Batterson, 1985). Only a few studies (Katz *et al.*, 1986; Zimber *et al.*, 1986; Katz *et al.*, 1988) have analyzed the restriction length polymorphism of EBV to study its molecular epidemiology, perhaps due to the difficulty of growing the virus.

Homology Between Herpesviruses and Human DNA:

Hybridization under stringent conditions has been noted between host cell DNA and the DNA of EBV (Hayward *et al.*, 1982) and HSV-1 (Peden *et al.*, 1982). Cross-hybridization between EBV or HSV DNA and cellular DNA may be due to an artifact: polypurine stretches will form complexes even in the absence of extensive sequence homology (Parks *et al.*, 1986). The internal repeat 3 (IR3) of EBV has homology to the human genome. The IR3 hybridizes to an interspersed repeat found in every human chromosome except the Y chromosome. Several of these homologous regions have been sequenced, but the significance of this homology is presently unknown (Dambaugh *et al.*, 1986).

Replication and Packaging of Herpesvirus DNA:

Herpesvirus DNA replicates semiconservatively in the nuclei of infected cells. The intracellular DNA forms a pool from which virion DNA is withdrawn at

random to be packaged and exported extracellularly (Roizman and Batterson, 1985).

A brief summary of the sequence of events which occurs during herpesvirus DNA replication in the cell nucleus is outlined below. After herpesvirus infection of a permissive cell, a series of sequentially coordinated events ensues, resulting in the production of progeny virions. Immediately after herpesviruses enter a cell, and before viral DNA synthesis occurs, the alpha genes (previously termed "immediate early genes") which encode for polypeptides 0, 4, 22, 27, and 47 are expressed, reaching a peak in their expression at 2-4 hrs postinfection. Because a *ts* mutant in alpha 4 accumulates only alpha polypeptides at nonpermissive temperatures, it can be concluded that the function of alpha 4 involves the induction of genes that are expressed later during the replicative cycle, the beta genes. The mechanism of regulation by the alpha 4 protein is unknown, but presumably it is a strong transducer of the beta genes. The amount of beta 1 and beta 2 gene products ("early antigens") peak at 5-7 hrs postinfection. Examples of beta 1 proteins are the viral ribonucleotide reductase and the major DNA binding protein; the thymidine kinase and DNA polymerase belong to the beta 2 polypeptide group. The beta gene products are the enzymatic machinery that performs the synthesis of viral DNA. The gamma gene products, which are the structural proteins (also known as "late antigens") may be of two types, 1 and 2. The gamma 1 genes (e.g., major capsid protein and glycoprotein B) are expressed before DNA synthesis, and the gamma 2 genes (e.g., glycoprotein C) require DNA synthesis for their expression (reviewed in Roizman and Batterson, 1985).

The rate of viral DNA synthesis, as determined by incorporation of labeled deoxynucleosides, appears to be highest relatively early in the infection. Newly synthesized viral DNA can be detected 3 hours following infection, with synthesis continuing for 9-12 hours (Roizman and Batterson, 1985). Ben-Porat (1977) estimated that the time required to replicate a unit-size HSV genome is approximately 20 minutes. Analysis of the molecular configuration of viral DNA in infected cells demonstrates that the viral genome lacks free ends, and, at least early in infection, most of the replication of DNA occurs in the episomal form, however, replication of some concatemers and unit-size linear molecules also occurs. The presence of circular molecules at the early stages of infection would suggest that there may be exonucleolytic digestion of the termini, followed by circularization or head-to-tail fusion of their cohesive ends (Ben-Porat and Veach, 1980). After circularization of the viral genome, replication is initiated at the origin(s) of replication. Replicating EBV DNA forms theta-type structures, at least during the first rounds of replication. Late in infection, replicated HSV DNA is in a rapidly sedimentable form of tangled DNA (Jongeneel and Bachenheimer, 1981). It thus seems likely that, early in infection, HSV and EBV DNAs replicate as episomes, and, during the late stages of infection, HSV DNA replicates by a rolling circle mechanism (McGeoch, 1987). However, further studies are necessary in order for the scheme of herpesvirus replication to be completely understood.

Newly synthesized DNA is packaged into preformed empty capsids. Before packaging, viral DNA lacking free ends (circular or head-to-tail concatemers) is cleaved into unit size molecules (a process known as "headful" encapsidation), although capsids may accommodate DNA molecules somewhat smaller or larger than the unit size. The genomes of all herpesviruses contain unique sets of end

sequences, which suggests that site-specific cleavage of concatemeric DNA occurs and that cleavage and/or encapsidation of the genome of concatemeric DNA is controlled by both site-specific recognition and "headful" encapsidation (Ben-Porat, 1981).

THE EPSTEIN-BARR VIRUS

General Biology of the Epstein-Barr Virus

The host range of EBV includes humans and other primates. Because of the excellent adaptation to its host, it is believed that EBV has been associated with humans for millions of years. EBV is able to persist in the human body despite the presence of circulating neutralizing antibodies and surveillance by specific cytotoxic T cells. Latent EBV infection, which is probably not detected by the immune system (Yao *et al.*, 1989), occurs in immature oropharyngeal cells which may be the immunologically privileged site in which EBV is able to persist in the latent state. Differentiated oropharyngeal epithelium is permissive for the replicative cycle of EBV and may be recognized by the immunosurveillance system. The oropharyngeal epithelium plays an important role in the spread of infection.

Background and Clinical Significance

Denis P. Burkitt made the initial observations that led to the discovery of the Epstein-Barr virus by Epstein, Achong, and Barr in 1964. Initially, Burkitt's lymphoma was described in Ugandan children affected with jaw tumors. An association between hyperendemic malaria and Burkitt's lymphoma was also found. Burkitt remarked: "In our early safaris, we had actually and unwittingly mapped out the geography of holoendemic malaria, rather than the geography of

the virus" (Burkitt, 1987). Subsequently, when it was found that malaria can be immunosuppressive, the reason for the association became apparent: under conditions of immunosuppression EBV can be oncogenic, leading to the onset of lymphoproliferative disorders (Burkitt, 1987). It was tissue from one of Denis Burkitt's lymphoma patients that was used for the initial isolation of the virus.

In 1968 Werner and Gertrude Henle described the cause-and-effect relationship between Epstein-Barr virus and heterophil-antibody-positive infectious mononucleosis (Henle *et al.*, 1968). Primary infection with EBV takes place either in childhood or in adolescence; thereafter the viral genome persists in human B lymphocytes, presumably in a latent state, throughout life.

The association between EBV and undifferentiated nasopharyngeal carcinoma is strong and constant in every part of the world. Most nasopharyngeal biopsies examined contain EBNA or EBV DNA (zur Hausen *et al.*, 1970). Reactivation of EBV latency in the nasopharynx in an appropriate host may be a vital step in the onset of nasopharyngeal carcinoma. Such reactivation is accompanied by the production of EBV-specific IgA (Henle and Henle, 1979).

Table I-1 lists the conditions under which EBV is believed to play an etiologic role.

More recently, a further association between a recent EBV infection and the onset of Hodgkin's lymphoma has been suggested by seroepidemiologic and nucleic acid hybridization studies. The Sternberg-Reed cell, which is pathognomonic of Hodgkin's lymphoma, contains EBV genomes (Weiss *et al.*, 1989).

Several biologic features distinguish EBV from other herpesviruses. For instance, only EBV is capable of infecting and immortalizing B lymphocytes to induce a polyclonal proliferation *in vitro* (Pope *et al.*, 1968). EBV causes experimental lymphoproliferative disorders in cotton-top tamarins (Frank *et al.*, 1976) and may result in a fatal lymphoproliferative disease in individuals with Duncan's syndrome, a congenital immunodeficiency with a principal defect in T cell immunity (Purtilo *et al.*, 1979). Organ transplant recipients who are iatrogenically immunosuppressed for long periods of time may develop EBV-associated lymphoproliferative disorders. Immunosuppressive drugs such as cyclosporin A have a profound effect on the cell-mediated immunity but little effect on the humoral arm of the immune system. Cyclosporin A suppresses the function of EBV-specific T cells both *in vivo* and *in vitro* (Leoni, 1978). The increase in viral shedding in oropharyngeal washings observed in organ transplant recipients receiving cyclosporin A and the inability to control EBV infection in patients with Duncan's syndrome suggest that the control of EBV replication is largely dependent on T cells. EBV genomes have been demonstrated in most tumor biopsies from patients with renal and cardiac transplants who develop lymphomas (Hanto *et al.*, 1981). It has been proposed that EBV infection of a circulating B cell containing a chromosomal translocation which deregulates the expression of the c-myc oncogene leads to the development of a clone of cells with fully malignant potential (Lenoir, 1982). Increased pharyngeal shedding of the virus, which may be associated with viremia or increased infection of B cells travelling through tonsillar tissue, may increase the number of transformed and actively multiplicating B cells and could increase the chances of such a malignant phenotype occurring.

Interaction of EBV and Human B Lymphocytes

Because EBV infection is a good experimental model in which to study the initial steps in oncogenesis, one of the most productive areas of EBV research is centered around the early stages of interaction of EBV and B cells. Virus binding to B lymphocytes occurs by a specific interaction between the major envelope glycoprotein gp340 and the receptor for the C3d component of complement (C3dR) (also known as "DC21" or "DR2") which is a 145-kDa surface glycoprotein (Nemerow *et al.*, 1987). C3dR expression on human lymphocytes is linked to B cell differentiation. Since the receptor is lost at the plasma cell stage of terminal differentiation, only earlier precursors of plasma cells are infected by EBV (Tedder *et al.*, 1984). EBV-associated diseases, such as Burkitt's lymphoma, infectious mononucleosis, and diffuse lymphoproliferative disorders, arise from infection of the B cell lineage. EBV induces normal B lymphocytes to proliferate *in vitro* and to become activated in long-term cell lines that can be propagated *in vitro* indefinitely (Pope *et al.*, 1968). The induction of indefinite B cell proliferation *in vitro* is termed "transformation" or "immortalization." Although infection of lymphocytes by EBV is a necessary step in achieving B cell activation and immortalization (Hutt-Fletcher *et al.*, 1983), it has been shown that the transfer of EBV receptors to EBV-receptor-negative cells, which allows the virus to infect cells and express some viral antigens, does not result in immortalization (Volsky *et al.*, 1981, 1984). This suggests that infection alone is not sufficient to induce immortalization. More recent evidence suggests that the EBNA-2 gene product is responsible for immortalization. About 10 of the approximately 100 genes of EBV are expressed in recently immortalized B cells, but experiments using a transformation-defective strain of the virus as a helper virus and fragments of EBV

DNA containing *cis*-acting elements suggest that only the gene encoding the EBNA-2 is required for immortalization of B cells (Hammerschmidt and Sugden, 1989). The EBNA-2 protein induces expression of the CD23 antigen (Wang *et al.*, 1987), which is a B cell activation signal that induces a receptor for a B cell growth factor (Gordon *et al.*, 1986).

Lymphocytes permissive for viral replication cannot be demonstrated *in vivo* (Rickinson *et al.*, 1975), but *in vitro* infection of B cells results in production of virions at very low levels. In clonal cell lines obtained by EBV transformation of adult lymphocytes, the frequency of cells releasing infectious EBV during a 24-hour period is one in 10^4-10^6 cells, and only 1 of every 10-30 EBV particles produced is able to infect B cells (Sugden, 1982). In the case of B95-8, an EBV-transformed marmoset cell line (Miller *et al.*, 1972), the percentage of cells releasing virus is approximately 5% at any one time. The number of EBV-infected B cells per unit volume of blood varies considerably among EBV-seropositive individuals. For a given individual the level remains relatively constant and may parallel the amount of virus shed from the oropharynx (Yao *et al.*, 1985). EBV may infect B cells that are in transit through the Waldeyer's ring or the lymphoepithelium of the postnasal space, which is in close association to oropharyngeal cells where EBV may be actively replicating. The characteristics of the B cell subset susceptible to EBV infection are still poorly defined. One study has suggested that only B lymphocytes with a high buoyant density can be induced to produce EBNA by EBV and become immortal, implying that resting lymphocytes are the predominant targets for EBV infection (Aman *et al.*, 1984). Under normal circumstances, there is a fine balance between EBV replication, B cell infection, and the humoral and cellular arms of the immune system. The

disruption of this balance by immunosuppression results in an increased pharyngeal shedding of EBV and in an increased number of infected B cells. It is possible that the lymphoproliferation associated with EBV infection is an effect of a dual process: immunosuppression resulting in decreased surveillance of EBV infected B cells, and increased replication of EBV in the oropharynx resulting in the infection of more B cells.

Interaction of EBV and Human T Lymphocytes

There is increasing evidence that EBV may infect T cells. One study suggested the presence of EBV receptors on T cells (Sauvageau *et al.*, 1987). Recently, purified CD4+ T cells from a child with chronic active EBV infection and a presumptive diagnosis of Kawasaki disease were found to harbor EBV (Kikuta *et al.*, 1988). The EBV genome has also been detected by *in situ* hybridization in T cell lymphomas of the CD4+ phenotype, which developed in patients with a chronic illness manifested by fever, pneumonia, dysgammaglobulinemia, hematolologic abnormalities, and high titers of antibodies to EBV antigens (Jones *et al.*, 1988).

An unusual cell line, termed "P3HR-1," produces an EBV variant which does not induce purified B cells to proliferate, but has a clear mitogenic effect on purified non-immune T cells. This cell line was obtained by cloning a Burkitt's-derived cell line (Jijoye). The mechanism of T cell proliferation induced by this variant remains unresolved (reviewed by Tosato and Blaese, 1985). It is possible that some cases of chronic mononucleosis syndrome are caused by a strain of EBV

that is defective and unable to induce immortalization of B cells. This could explain why some individuals with the syndrome have increased T suppressor cells and do not have increased numbers of transformed B cells (Tosato and Blaese, 1985). Alfieri *et al.* (1987) found evidence which supports this hypothesis by isolating a P3HR-1-like virus from patients with the chronic mononucleosis syndrome.

EBV in the Oropharynx

Oropharyngeal epithelial cells provide the target for the replicative cycle of EBV in acute infectious mononucleosis and in the chronic carrier state (Diaz-Mitoma *et al.*, 1987). EBV DNA and RNA can be demonstrated in oropharyngeal epithelial cells by *in situ* hybridization. A receptor for EBV, which is not homologous to the C3dR in lymphocytes, has been identified on human pharyngeal epithelial cells. This molecule is a 200-kDa surface protein that shares some epitopes with the C3dR of B lymphocytes (Young *et al.*, 1989). Infectious virions can be detected (by lymphocyte transformation) in the saliva of individuals who acquire primary infections with EBV. Several studies have shown that 15-27% of EBV-seropositive individuals in developed countries shed EBV in oropharyngeal secretions (Gerber *et al.*, 1972; Golden *et al.*, 1973; Diaz-Mitoma *et al.*, 1987; Sixbey *et al.*, 1989). Other studies suggest that almost all EBV-seropositive individuals shed some virus, with the levels of excretion remaining constant in specific individuals over long periods of time (Yao *et al.*, 1985).

The expression of viral antigens indicative of a productive infection, such as early and viral capsid antigen, and virion production seems to be restricted to the uppermost layers of cells in the stratified tissue and recently desquamated cells (Sixbey *et al.*, 1983). Productively infected epithelial cells are no longer mitotically active, but are mature, terminally differentiated cells. The only gene product detected in the mitotically active cells of the basal and parabasal layers is the nuclear antigen complex (EBNA) (Sixbey *et al.*, 1983). Since there is evidence that EBNA-1 antigen is sufficient for maintenance of the episomal form of EBV (Yates *et al.*, 1985), immature cells infected with EBV may restrict the expression of viral genes to the minimum required for replication of episomal forms of EBV DNA. It is not known whether the immune system is able to recognize these cells, with their restricted expression of viral genes during latency.

Replication of EBV seems to be closely associated with the differentiation of epithelial cells. EBV infects receptor-positive, immature cells of the epithelium, which differentiate and become permissive for virus replication. The latent infection of the immature basal and parabasal cell layers in the epithelium of the oropharynx forms a permanent pool of self-renewing EBV-infected cells. This strategy is compatible with EBV persistence and continuous shedding of virus.

EBV in the Uterine Cervix

Until recently, sites for EBV replication other than the oropharynx had not been identified. *In vitro* and *in vivo* studies suggest that EBV replicates in the

epithelium of the uterine cervix. Sixbey *et al.* (1983) were able to infect cervical epithelial cells with EBV, and the virus was detected in 5 out of 28 women by means of culture and cytohybridization analysis of cervical secretions (Sixbey *et al.*, 1986). The epithelial EBV receptor has been identified in cervical epithelium as well as in oropharyngeal cells. It is preferentially expressed in immature, metabolically active, and less differentiated cells of the basal and parabasal layers of the cervical epithelium (Sixbey *et al.*, 1986, 1987). In addition, EBV may also replicate in other genital sites. Portnoy *et al.* (1984) reported the recovery of infectious EBV from a genital ulcer in a patient with infectious mononucleosis. The presence of EBV in cervical secretions could suggest venereal and/or perinatal transmission of EBV.

Cytopathic Effects Induced in the Epithelium by EBV Infection

A limiting factor in the study of the replicative cycle of EBV in the epithelium has been the lack of a permissive cell line. Recently, Saito *et al.* (1989) studied the cytopathic effect of EBV in an epithelial-nasopharyngeal carcinoma hybrid cell line (cl. S61). These cells produce virions and demonstrate cytopathic effects similar to the cytopathic changes associated with replication of other herpesviruses, including cell rounding and the formation of multinucleated giant cells, nuclear inclusions, and cytoplasmic inclusion bodies. A virus-specified glycoprotein is probably responsible for cell fusion because 2-deoxyglucose (an inhibitor of glycosylation) interferes with syncytium formation (Saito *et al.*, 1989).

Anatomy of the EBV Genome

The genome of EBV virions is a linear, double stranded DNA molecule that measures 172 kbp (see Figure I-3). The entire genome of EBV has been cloned (Dambaugh *et al.*, 1980; Raab-Traub *et al.*, 1980) and sequenced (Baer *et al.*, 1984). The genome contains non-random single-strand breaks (Pritchett, 1975) and consists of unique and tandemly repeated DNA elements. The number of tandem repeats varies among different EBV isolates and among molecules from each isolate (reviewed in Dambaugh *et al.*, 1986). Figure I-2 represents a detailed genomic map of EBV DNA organized in the following sequence: TR-U1-IR1-U2-IR2-U3-IR3-U4-IR4-U5-TR. TR or terminal repeats are direct, 550 bp repeats located in the telomeres of the genome (Given *et al.*, 1979). The IR1 (internal repeat 1) consists of direct, tandem, 3072 bp repeats; IR2 (internal repeat 2) contains direct 125 bp repeats; and IR4 (internal repeat 4) is composed of 103 bp repeats (Cheung and Kieff, 1982; Dambaugh and Kieff, 1982). IR3 is the internal repeat 3, and U1, U2, U3, U4, and U5 are unique DNA elements.

Structure and Function of the Termini of the DNA of EBV

After infecting B lymphocytes, EBV persists in a latent state. It is thought that, upon entry into the cell, the linear EBV genome circularizes by the joining of the 550 bp tandem repeats of the TR region, producing a fused terminal restriction fragment. It is not known whether the circularization process involves homologous recombination or end-to-end ligation of terminal repeats which had

been cleaved with a specific endo- or exonuclease in the process of encapsidation. The EBV genome remains latent in B cells as covalently closed circular episomes (Lindahl *et al.*, 1976). A linkage map of the fragments generated by cleavage of virion DNA with restriction endonucleases indicated that heterogeneity exists in the molecular weights of the restriction fragments containing the terminal repeats (Given and Kieff, 1978). This heterogeneity results from a variation in the number of units of the terminal repeat present in different molecules of viral DNA. About 70% of the DNA molecules isolated from virions of the B95-8 strain of EBV have four terminal repeats at one end, while four equal classes, each comprising 25% of the population, have one, two, three, or four repeats at the other end. The fragments produced by endonucleases and assumed to be terminal in virion DNA are absent from intracellular circular DNA. The molecular weight of the fragment found in intracellular DNA was equivalent to the molecular weights of the two terminal fragments which are joined upon intracellular circularization of viral DNA (Kintner and Sugden, 1979). Because exonucleolytic digestion of the termini could result in progressively shorter genomic molecules after several rounds of replication, regeneration of the missing terminal sequences after cleavage of unit size molecules is required. The simplest mechanism by which these end sequences could be regenerated is by creating a staggered cut in the junction between the molecules, thus producing free 3' ends. The resulting single-stranded regions are then repaired by DNA polymerase. However, this could not explain the heterogeneity found by Kintner and Sugden in B95-8 DNA. Other strategies of DNA replication may explain the heterogeneity of the TR in herpesvirus DNA, such as blunt-end ligation of the repeats and strand migration from the terminus into the terminal segment of another molecule with terminal repeats (reviewed by Morgan and

Severini, 1989). It is likely that the TR of EBV may play a role in the maintenance of latency and in the recognition sites for cleavage and packaging. It is similar in many respects to the herpes simplex virus (HSV) terminal repeat "a" sequence, a repeat element which is specifically recognized for cleavage and packaging (Mocarski and Roizman, 1981). Both sequences are G-C rich, have oligo dG and oligo dC domains, and are bracketed by short terminal direct repeats. As well, within TR near the short EBV terminal direct repeat is a sequence GGCCCCCAGGAAAAGACCCCCGG which has homology to the highly conserved domain of the HSV "a" sequence which could be the HSV DNA cleavage recognition site (Dambaugh *et al.*, 1986).

Several studies have suggested that the structure of the termini of the EBV genome may define the clonotypic origin of B cell clones (Raab-Traub and Flynn, 1986; Brown *et al.*, 1988; Katz *et al.*, 1989). Analysis of the fused termini of EBV DNA in 17 B cell lineages cloned from two patients with infectious mononucleosis demonstrated variation in the size of the termini in all different cell clones isolated from the same patient. EBV termini differed by as many as 15-20 reiterations of the 550 bp terminal repeat sequence. In comparison, analysis of seven B cell lineages cloned from a patient with a fatal, oligoclonal lymphoma revealed that three of the cell clones had the same-sized EBV terminal region. These clones were shown to have been descended from a common progenitor cell by immunoglobulin gene analysis (Brown *et al.*, 1988). Similarly, Raab-Traub and Flynn (1988) analyzed the configuration of the termini of the viral genome and found that the permissive cell line B95-8 contained termini with free ends, representing linear EBV genomes, and three types of fused termini, suggesting a polyclonal proliferation. In contrast, the cell line Raji and tumoral tissues, such as

nasopharyngeal carcinoma and lymphoma, contained only one type of fused termini, suggesting a monoclonal proliferation. It is thought that, upon entry into a susceptible cell, the EBV genome circularizes via its terminal repeats, is amplified in copy number, and resides in the cell nucleus mostly as a multicopy plasmid, and perhaps with integrated copies as well. *In situ* hybridization studies of the Namalwa Burkitt lymphoma cell line and a lymphoblastoid cell line indicated that EBV DNA integrates at specific chromosomal sites. EBV DNA co-localized with a stable achromatic gap at 1p32 in the lymphoblastoid cell line transformed *in vitro* with EBV. The achromatic gap conferred a proliferative advantage to the cells. The Namalwa and the lymphoblastoid cell lines contained the same five chromosomal bands which demonstrated EBV DNA (Caporossi *et al.*, 1988). Studies of the terminal repeats of the EBV-positive cell line IB4 (derived from human lymphocytes inoculated with the B95-8 isolate of EBV) suggest that the EBV genome is integrated into cellular DNA. Since unique-sequence DNA from the left and the right ends of the EBV genome are linked in all IB4 EBV DNA molecules, integration into IB4 cells is likely to have been from an episomal intermediate. This may suggest that the TR is also involved in the integration of circular DNA into the cellular genome.

Replication of EBV DNA

The replication of EBV is under the control of viral and cellular genes. EBV has two distinct life cycles: a latent and a lytic (also called "permissive infection") cycle. During latent infection only episomal forms of the EBV genome are found intracellularly (Lindahl *et al.*, 1976). The episomes of EBV DNA are replicated

early during the S phase of the cell cycle (Hampar *et al.*, 1974). Viral DNA in latently infected cells is probably replicated by cellular polymerases, since EBV DNA replication in latently infected cells is resistant to phosphonoacetic acid and acyclovir, while viral DNA polymerase is sensitive to phosphonoacetic acid and acyclovir (Colby *et al.*, 1980). Episomal EBV DNA appears to replicate by the theta structure model. The presence of relaxed theta structures with more than two replication forks suggests that there may be more than one origin of replication (Dambaugh *et al.*, 1986); however, studies of plasmid survival in EBV-infected cells identified only one origin of replication (oriP) in the EBV genome (Yates *et al.*, 1985). During latency, the *cis*-acting DNA sequence oriP and the *trans*-acting protein EBNA-1 are necessary elements for plasmid replication. EBNA-1 likely activates episomal replication through an interaction with cellular factors (Wysokenski and Yatz, 1989) and viral DNA is replicated by the cellular polymerase (Sixbey and Pagano, 1985). In contrast to latent infections, the DNA sequence element oriLyt and the viral DNA polymerase mediate the replication of the EBV genome during the viral lytic cycle (Hammerschmidt and Sugden, 1988). Latently infected B cells usually contain more than one copy of the EBV genome (zur Hausen *et al.*, 1970). In addition to the control of episomal replication perpetrated by EBNA-1, cellular gene products such as the B-cell growth factor (BCGF-12 kDa) produced by T cells down-regulates the replication of episomal DNA in Burkitt lymphoma cells (Raji) and in lymphoblastoid cell lines. (Morgan *et al.*, 1989).

Heterogeneity of the EBV Genome and Typing of EBV Strains

The wide spectrum of clinical entities associated with EBV infection has led to the hypothesis that specific strains of EBV might be responsible for particular conditions (Pizzo *et al.*, 1978). This hypothesis has been a central theme in EBV research and has led to important clarifications of the structure of the virus, but, until recently, it had failed to demonstrate a unique association between a certain strain and a certain disorder (Raab-Traub *et al.*, 1980). Early studies established that there is at least a 90% homology between EBV DNAs of different origin (Kawai *et al.*, 1973; Pritchett *et al.*, 1975). Analysis of viral genomic DNA by restriction endonuclease length polymorphism has indicated several differences among various EBV isolates. For instance, the B95-8 EBV variant has a deletion involving approximately 10 MDa of its DNA when compared to EBV DNA from Burkitt's lymphoma isolates or from different acute IM isolates (Fischer *et al.*, 1981; Katz *et al.*, 1988). In addition, P3HR-1 DNA is heterogeneous and consists of at least two different sets of molecules (Miller *et al.*, 1985). These studies have failed to correlate a specific DNA sequence with a particular type of disease, but have suggested that EBV may be transmitted from mothers to infants. Similar restriction endonuclease patterns are found in EBVs isolated from mother-infant pairs (Katz *et al.*, 1986, 1988). Katz *et al.* (1986) have also demonstrated that some individuals may be infected with two variants of EBV and that EBVs isolated from unrelated individuals could also be differentiated from each other. They also found the same strains of EBV in saliva and in peripheral blood by isolating immortalizing EBV, which is evidence that viral strains that replicate in the oropharynx are the reservoir for ongoing infection of lymphocytes. However, this

finding does not exclude the hypothesis that "purely replicative," non-immortalizing strains of EBV may replicate in the oropharynx (Alfieri *et al.*, 1987).

The heterogeneity of the DNA sequence of the EBNA-2 gene has been useful in the typing of EBV isolates. Striking differences in the EBNA-2 encoding region have been found in isolates from Central Africa and strains isolated from North America. EBV isolates may be of two types, according to the structure of the EBNA-2 gene or protein, types A and B. EBNA-2A has been found in healthy EBV-seropositive individuals in the United Kingdom and Australia (Young *et al.*, 1987) In contrast, EBNA-2B has been found in up to 40% of Burkitt's lymphoma cell lines, in the peripheral blood lymphocytes of 20% of adults in Africa (Zimber *et al.*, 1986; Young *et al.*, 1987), and in 40% of patients with HIV infection (Sculley *et al.*, 1989). The EBNA-2 protein is encoded by the *BamH*I-H fragment of the EBV genome. The EBNA-2 gene can be used to characterize EBV isolates based on the high divergence, at both the protein and DNA levels, between the type A and type B variants. The ability to transform B cells also differs between these variants, type B (such as BL16 and AG876) transforming B cells less efficiently than type A (such as the B95-8 strain). In contrast to previous reports, Sixbey *et al.* (1989) found that infection by the type B variant is widely distributed in healthy EBV-seropositive individuals in North America. This study demonstrated that, of 34 individuals excreting EBV DNA in throat washings, 14 (41%) were shedding type B virus, 17 (50%) were shedding type A, and 3 (9%) were infected by both strains of the virus. The same authors have also suggested that type B strains are involved in the pathogenesis of hairy leukoplakia in HIV-seropositive individuals.

Expression of Latent EBV Genomes

When EBV infects a cell, two mutually exclusive events may ensue, either the establishment of a latent infection resulting in immortalization of B lymphocytes, or viral replication resulting in the production of progeny virions and probably death of the host cell. Latently infected lymphocytes and probably also EBV-infected immature epithelial cells express at least four EBV proteins. These proteins are encoded in regions of the EBV genome that are designated LT1, LT2, LT3, and LT4, or latent cycle transcripts 1, 2, 3, and 4 (reviewed by Dambaugh *et al.*, 1986) (see Figure I-3). The LT1 gene is contained in the IR1 and U2 domains (Hennessy and Kieff, 1985). The LT1 gene codes for the EBNA-2 protein, this divergent gene being the basis for the differentiation between the two families of EBV variants discussed above (Zimber *et al.*, 1986). Differences among isolates of EBV in U2 at the DNA level correlate with differences in the 75-88 kDa nuclear protein detected by Western blotting (Sculley *et al.*, 1988; Sixbey *et al.*, 1989). In addition to the effects on the growth phenotype of transformed cells (Rickinson *et al.*, 1987), EBNA-2 mediates the human leukocyte antigen (HLA) class I antigen-restricted cytotoxic T cell response to EBV infection (Moss *et al.*, 1988). EBNA-1 is encoded in the LT2 gene which includes the right end of U3, IR3, and the left end of U4 (Figure I-3). An important biologic function of EBNA-1 is the maintenance of EBV episomes (Yates *et al.*, 1985). EBNA-1 may be similar to SV40 T antigen in its specific recognition of an origin of replication. Since EBNA-1 binds diffusely to chromatin, it may also have a direct effect on cellular origins of DNA synthesis (Dambaugh *et al.*, 1986). The LT3 is within U5 near the TR (Hudson *et al.*, 1985). The most abundant mRNA in the latent state, which is a 2.8 kb transcript, is encoded by this region. Two related membrane

proteins may be expressed by the reading frames of this mRNA. Among EBV isolates, polymorphism in LT3 depends on the number of reiterated sequences within U5, which correlates with polymorphism in size with a 60 kDa protein (Hennessy *et al.*, 1984). This protein could be responsible for recognition of EBV-infected cells by T cells [latent membrane protein (LMP)]. The LMP induces anchorage-independent and tumorigenic growth (Hammerschmidt *et al.*, 1989). LT4 is located to the left of LT2 within the U3 region. It encodes for a protein of 140 kDa which is translated from a 4.5 kb RNA. This protein is expressed in the nucleus of infected cells and has been designed as EBNA-3. This protein is also recognized by cytotoxic T cells and may be of three types, a, b, and c (Rowe *et al.*, 1989). There are at least six different EBNAs, which may not all be necessary for maintenance of the transformation phenotype because some cell lines do not express EBNA-3, -4 and -6, despite their immortalized phenotype (Rowe *et al.*, 1989). A fifth region, expressed during latency, encodes two small non-polyadenylated RNAs which may be involved as co-factors in the expression of late antigens (Bhat and Thimmapaya, 1983).

Disruption of EBV Latency

The production of virions can be increased by exposure of latent EBV-infected cells to short-chain fatty acids (Luka *et al.*, 1979), indole alkaloids (Ito *et al.*, 1981), tumor promoters such as phorbol esters, and some nucleoside analogs (Gerber, 1972). Upon treatment with 12-0-tetradecanoylphorbol-13-acetate, the 68 kDa EBNA-1 is uncoupled from the binding of the EBV origin of replication (oriP). Two proteins that compete for and uncouple the binding of EBNA-1 to oriP

are produced after phorbol ester stimulation (Wen *et al.*, 1989). The control of EBV replication and latency may also be dependent on the immune system. A B cell growth factor of ~12 kDa (BCGF-12 kDa) which is produced by T-cells down-regulates EBV episomes (Morgan *et al.*, 1989). The down-regulation of EBV episomes by BCGF-12 kDa raises the possibility that decreased levels of BCGF-12 kDa during immunosuppression may induce disruption of EBV latency.

Productive or Lytic Cycle

In latently infected cells, 8-10 EBV genes are expressed; in contrast, as many as 100 genes are expressed during the lytic cycle. Although most of the regulatory factors that determine whether an EBV infection will develop into latency or a lytic life cycle are unknown, an EBV immediate early protein of 34 kDa may play a regulatory role in lytic infection. This protein alone can disrupt latency by its transactivating functions (Kenney *et al.*, 1989). EBV-induced early antigens (EA) are detected in oropharyngeal cells or B lymphocytes producing viral particles. This group of antigens is detected early in the virus replicative cycle. EA have been further subdivided into diffuse (D) or restricted (R) components based on morphological staining patterns and sensitivity of these patterns to methanol fixation. The antigenic structures that are responsible for the R-type staining are denatured by methanol, whereas the D components are stable to treatment with methanol or acetone. Early antigen expression is not altered by the presence of inhibitors of viral DNA synthesis. The major proteins associated with the EA complex have molecular weights of 47-60, 85, and 140 kDa. Information on the possible role of these proteins is limited. It has been suggested that the EA

complex proteins are used in the early stages of replication of EBV DNA (protein kinase, DNA polymerase, DNAse, endonuclease, and thymidine kinase). Viral capsid antigens (VCA) and the EBV-induced membrane antigens (MA) are classified as late antigens, since their synthesis is inhibited in the presence of inhibitors of viral DNA synthesis. The major polypeptides comprising the viral capsid antigen complex include proteins of molecular weights of 125, 152, and 160 kDa. The MA complex has been studied extensively because of the interest in developing a subunit vaccine against EBV. MA are expressed in the envelopes of mature virions and are responsible for the induction of neutralizing antibodies (Pearson and Qualtière, 1978). The MA complex consists of three glycoproteins designated gp85/90 (glycoprotein with a molecular weight of 85-90 kDa), gp200/250 (glycoprotein with a molecular weight of 200-250 kDa), and gp300/350 (glycoprotein with a molecular weight of 300-350 kDa). Target antigenic determinants for neutralizing antibodies are expressed in all three major MA glycoproteins (Qualtière *et al.*, 1982). The EBV-induced nuclear antigens (EBNAs) are expressed in every cell containing the viral genome and therefore serve as an immunological marker for the viral DNA.

Immune Response to EBV Infection

Host immune mechanisms play a central role in the control of EBV infection. Numerous studies support the view that cellular immunity mediated by T lymphocytes is of primary importance for the control of B cells endogenously infected with EBV during initial infection (reviewed by Tosato and Blaese, 1985). Patients affected by congenital or acquired immune deficiencies are at an

increased risk of developing lymphoproliferative disorders secondary to EBV. A common characteristic of individuals at high risk of EBV-associated lymphoproliferation is a profound defect in the cell-mediated immune response (Crawford *et al.*, 1981).

Cell-Mediated Immune Response to EBV:

Attempts to explain how the immune system deals with EBV infections have focused on two main areas: the study of the acute immune response during infectious mononucleosis, and the study of the control and regulation of EBV-induced lymphoproliferation *in vitro* by T cells from EBV-seropositive individuals.

Acute infectious mononucleosis is characterized by the appearance of atypical lymphocytes in peripheral blood and infiltrating some tissues (Carter, 1975). Most of these cells are T lymphocytes reactive to the viral infection (Sheldon *et al.*, 1973). Analysis of the T cell subsets of atypical lymphocytes has demonstrated expansion of the cytotoxic/suppressor (T8+) phenotype and inversion of the helper:suppressor ratio (T4:T8) (Reinhertz, 1980). The T cells isolated from patients with infectious mononucleosis exhibit suppressor activity over pokeweed mitogen-activated B cells *in vitro* (Tosato, 1979). This is a polyclonal, broad suppressor T cell function, which is neither virus-specific nor preferentially active against activated B lymphocytes and may explain the general depression of cell-mediated immune activity observed in infectious mononucleosis (Rickinson, 1986). In addition, this population of polyclonally activated T cells contains cytotoxic cells which lyse infected cells expressing a viral antigen termed "lymphocyte-determined membrane antigen" (LYDMA) (Svedmyr and Jondal, 1975). A feature that distinguishes this cytotoxic response from other virus-specific cytotoxic responses is the lack of HLA restriction (Seeley

et al., 1981). Because natural killer cells (NK) have a broad-range cytotoxic activity, they could be the effectors of the cytotoxic response. However, patients with Chediak-Higashi syndrome, who lack NK cells, demonstrate a normal non-HLA-restricted cytotoxicity to EBV-infected cells; therefore it is unlikely that NK cells are the main effectors in the cytotoxic response during infectious mononucleosis (Katz *et al.*, 1984).

After the acute stage of IM, the EBV-induced lymphoproliferation is controlled by the immune system; EBV remains latent in B cells and the epithelium, and the asymptomatic virus carrier state is established. The components of the T cell response that control the lymphoproliferation of B cells infected by EBV were studied by analyzing the T cell functions induced *in vitro* by EBV-infected cells (reviewed by Rickinson, 1986). There are four basic mechanisms by which the cellular immune response controls EBV-induced lymphoproliferation. First, it was noted that, when T cells were added to cultures of infected B cells, there was delayed appearance of EBNA-positive cells, cell proliferation, and cell line outgrowth (Thorley-Lawson, 1980). This phenomenon occurs in EBV-seronegative and EBV-seropositive T cell donors and may be mediated by interferon gamma (Andersson *et al.*, 1983). Secondly, the activity of virus-specific cytotoxic cells (memory T cells) was demonstrated by observing their regulatory control over virus-induced transformation of B cells *in vitro* (Rickinson, 1986). Cells from seropositive donors allow the proliferation of EBNA-positive cells during the first 7 to 14 days, but this is followed by a regression of growth, destruction of EBNA-positive cells, and complete abrogation of cell line establishment. This cell response is virus-specific and is restricted by class I HLA antigens (Rickinson, 1986). The suppression of such immune response during cyclosporin A or other immunosuppressive drug

therapy may result in lymphoproliferative disorders. Thirdly, adult fibroblasts from EBV-seropositive individuals demonstrated inhibition of growth of EBV transformed cells, while fetal fibroblasts did not, which suggests an immune mechanism. Although the effector mechanism of this inhibition is unknown (Moss *et al.*, 1977), it is possibly mediated by lymphokines. The fourth regulatory control over EBV infection is mediated by late suppressor T cell activity, which inhibits immunoglobulin synthesis in EBV transformed B cells (Tosato and Blaese, 1985). Impairment of late suppressor activity may result in autoimmunity due to the outgrowth of forbidden clones of B cells which produce antibodies against self antigens (Shore *et al.*, 1989).

Humoral Response to Primary EBV Infection:

The humoral response during primary EBV infection is directed towards viral and cellular antigens. A list of antibodies identified in patients with primary EBV infection is shown in Table I-2. These antibodies are grouped into two categories, major and minor. Major antibodies are found frequently in patients with EBV infection and may be of diagnostic importance. Minor antibodies are unusual and do not have diagnostic relevance.

Heterophil antibodies: Confirmation of the diagnosis of IM largely depends on the demonstration of these antibodies in the patient's serum. The heterophil antibody is an IgM that agglutinates sheep, bovine and horse erythrocytes, but fails to agglutinate guinea pig erythrocytes. Most commercial tests such as the Monospot test (Ortho Diagnostics, Raritan, N.J.) use horse red cell agglutination as an indicator for infectious mononucleosis-specific heterophil antibodies following absorption with guinea pig red blood cells. Approximately 90-95% of individuals with IM develop heterophil antibodies. Only 2% of the general

population has detectable heterophil antibodies (Evans *et al.*, 1975). Children are less likely to develop heterophil antibodies after IM. These antibodies are detected just preceding or during the acute phase of IM, decreasing to undetectable levels three to six months postinfection. Maeda *et al.* (1979) suggested that the heterophil antigen is expressed in EBV-infected cells, but is not an EBV antigen. The production of heterophil IgM antibodies after EBV infection may be explained by the fact that B cell precursors committed to IgM production have a greater susceptibility to EBV infection than those committed to IgA or IgG production (Tosato and Blaese, 1985).

Antibodies to EBV-associated antigens: The detection of antibodies to specific EBV antigens is useful for identifying the small percentage of individuals that develop heterophil-negative IM-like disorders caused by EBV and may also distinguish EBV-induced IM from malignant diseases having features in common with IM. Viral capsid antigens (VCA) are late antigens identified in virus producing cell lines. Currently the most useful test for the serologic diagnosis of IM is the IgM antibody to the viral capsid antigen. These antibodies appear early during the acute phase of infection, but disappear soon after the acute illness. VCA-IgM antibodies are detectable in about 97% of patients during the acute phase of IM (Evans *et al.*, 1975). The VCA-IgG is used to determine whether a person has been infected with EBV. VCA-IgG antibodies appear early after the onset of IM, and it is difficult to demonstrate rising antibody titers unless the first serum is obtained very early during the acute phase of the illness. Because these antibodies persist at a high titer for a long period, they have only limited diagnostic value.

Cells expressing only early antigens (EA) can be produced by cultivating virus-producing lymphoblastoid cell lines in the presence of inhibitors of DNA

synthesis, such as phosphonoacetic acid (Pearson, 1983). Two types of early antigens have been described. The first is the diffuse or D type and is detected in both the nucleus and the cytoplasm of infected cells. The second early antigen is termed "restricted" or R type and is detected only in the cytoplasm. Antibodies to the D component are detected in 70-80% of patients with acute IM. EA-IgG antibodies appear early during acute illness and decrease to undetectable levels during the convalescent phase of the illness. Persistence of antibodies to EA is thought to be an indication of an active EBV infection (Henle and Henle, 1978). However, immunosuppressed patients and some non-immunosuppressed individuals do not demonstrate EA-IgG antibodies during active EBV infection (Ho *et al.*, 1985), and some asymptomatic individuals who do not have detectable EBV in throat washings demonstrate persistently high titers of EA-IgG antibodies (Diaz-Mitoma *et al.*, 1987). There are obvious limitations for the use of EA-IgG antibodies in the diagnosis of active or reactivated EBV infections. The detection of the EBV DNA in oropharyngeal secretions may be a more direct and accurate indicator of active EBV infection than the detection of EA-IgG antibodies (Diaz-Mitoma *et al.*, 1987).

The EBV-induced nuclear antigens or EBNAs were initially demonstrated using the anticomplement immunofluorescence procedure. Antibodies against EBNA are usually detected weeks after the primary infection. There are six distinct EBV-nuclear antigens (EBNA-1 to EBNA-6). Following IM, antibodies to EBNA-2 are the first to appear, reaching peak titers after several weeks, and then declining to low or undetectable levels. Antibodies to EBNA-1 emerge several weeks or months after anti-EBNA-2 and gradually rise and persist indefinitely. The ratios between the anti-EBNA-1 and anti-EBNA-2 titers are below 1.0 during the first 6-12 months after IM and increase to well above 1.0 during the second

year. It has been suggested that, in cases of chronic IM, the inversion of the ratio is delayed or prevented (Henle *et al.*, 1987); however, the anti-EBNA-1 to anti-EBNA-2 ratios have not been studied in detail, and further confirmation of their diagnostic value is necessary.

Antibodies against the MA complex have the greatest potential to influence the course of infection because of their neutralizing properties. It is believed that neutralizing antibodies play a central role in preventing viremia during reactivation and prevent reinfection on exposure to EBV (Henle and Henle, 1979; Rickinson *et al.*, 1975).

Diagnosis of EBV Infection

Exposure of a previously uninfected host to EBV may result in infectious mononucleosis (IM) or in subclinical infection. The diagnosis of primary EBV infection is based on a typical clinical picture, characteristic hematologic changes, and a serologic response. Young adults develop a triad of symptoms, which includes sore throat, fever, and lymphadenopathy. Other manifestations are periorbital edema, palatine exanthem, splenomegaly, and jaundice. The presence of elevated liver enzymes is also a valuable diagnostic feature. The second criterion for the diagnosis of IM is lymphocytosis with atypical lymphocytes in the peripheral blood (>10% of the total leukocyte count). The third criterion required for diagnosis of IM is the demonstration of an antibody response to EBV. Although the diagnosis of infectious mononucleosis does not usually present a problem, EBV infection, especially when it is severe, can mimic a wide spectrum of diseases such as acute leukemia or disseminated lymphoma,

familial lymphohistocytosis, idiopathic aplastic anemia, common variable immunodeficiency, severe hepatitis, and, more rarely, multiple sclerosis and some autoimmune diseases (Purtilo *et al.*, 1984).

Detection of EBV Infection by Tissue Culture:

The "gold standard" for identification of viruses in clinical specimens is tissue culture whereby infectious EBV virions are detected by their ability to cause transformation of B lymphocytes. Infection of lymphocytes may be confirmed by detection of EBNA by immunofluorescence. EBV is detected in oropharyngeal secretions of patients with IM, in EBV-seropositive immunosupressed patients, and in some asymptomatic EBV-seropositive individuals. Ten to 60% of asymptomatic EBV-seropositive individuals and 90-100% of heterophile-positive IM patients shed EBV in the oropharynx, as detected by the lymphocyte transformation assay. Similarly, the virus may be demonstrated by co-cultivating patients' tissues with lymphocytes from umbilical cord blood or by isolating cell lines *in vitro* from lymphoid tissues of patients with suspected EBV-infections. There are several factors which influence the detection of EBV by the lymphocyte transformation assay, including the EBV-serostatus of the B cell donor, the transforming activity of the EBV strain, the history of previous EBV infections, the time since a primary EBV infection, the integrity of the immune system, the levels of virus excreted, and the *in vitro* conditions used in the assay. Several important factors should be considered when lymphocyte transformation is used to detect EBV. Firstly, fetal cord lymphocytes or lymphocytes from EBV-seronegative donors should be used for the assay since lymphocytes from EBV-seropositive individuals may contain EBV-infected cells or memory T cytotoxic cells specific for EBV-infected cells, which may inhibit the outgrowth of transformed lymphocytes.

Secondly, transformation-defective EBV strains may not be detected by this system. For example, Alfieri *et al.* (1987) described a family in which a transformation-defective strain of EBV was identified. This EBV isolate was a P3HR-1-like isolate, which is an unusual variant that contains a deletion in the U2 region of the EBV genome. As discussed previously, the EBNA-2 protein, which is encoded in this region, is responsible for the immortalized phenotype. In addition, Sixbey *et al.* (1989) found that approximately half the individuals in whom EBV DNA was demonstrated in oropharyngeal secretions were negative for EBV by the lymphocyte transformation assay, and most of the EBV DNA-positive, lymphocyte transformation-negative individuals were infected by an EBV variant that was transformation-defective. There is some evidence that this transformation-defective variant or type B virus is widespread in North America (Sixbey *et al.*, 1989). A third factor which influences the excretion of EBV in the oropharynx is the length of time elapsed since the primary EBV infection. The oropharyngeal shedding of EBV may persist at low levels in asymptomatic individuals for weeks or months after an initial episode of IM. We have followed patients who excrete EBV for up to three years after IM. Asymptomatic, EBV-seropositive patients who are immunodeficient have a high rate of EBV excretion. It is unknown whether the levels of oropharyngeal excretion of EBV have predictive value at the onset of illness in immunosuppressed individuals. Theoretically, the lymphocyte transformation could detect as few as 30 virions, since 1 in every 30 virions is infectious (Sugden, 1982). Nevertheless, individuals excreting low or high levels of the virus will not be differentiated by lymphocyte transformation unless a limiting dilution assay of the transformed cells is used to quantitate the level of virions excreted. This assay would not take into account the degree of transforming activity of different EBV strains. Another

important consideration when performing cultures of peripheral lymphocytes from EBV-seropositive donors is the presence of a feeder layer of fibroblasts, which may provide growth factors necessary for the *in vitro* outgrowth of lymphoblastoid cell lines. Another problem with the lymphocyte transformation assay is that it is time-consuming, taking 3 to 6 weeks to demonstrate the virus in clinical specimens. Because of the limitations in the performance and interpretation of the lymphocyte transformation assay, newer methods for the detection of EBV are needed.

Detection of EBV Infection by Nucleic Acid Hybridization:

Nucleic acid hybridization represents a sensitive and specific method of demonstrating virus-specific DNA in lymphoblastoid cell lines (Brandsma and Miller, 1980). The development of cloned subgenomic fragments of EBV has made new reagents available for the detection of EBV in clinical specimens. DNA probes containing the *Bam*HI-W repeating subgenomic fragment of EBV have been used to detect the virus genome in lymphoma tissue (Ho *et al.*, 1985), bronchial cells (Lung *et al.*, 1985), and exfoliated epithelial cells in patients with nasopharyngeal carcinoma (Pi *et al.*, 1983). Some of the features of the nucleic hybridization system that differ from those of the lymphocyte transformation assay are that non-infectious viral DNA and non-transforming variants of EBV may be detected. In addition, clinical specimens under long-term storage may be analyzed for the presence of viral DNA, and nucleic hybridization techniques allow for a detailed analysis of the genomic structure of the EBV.

EBV Infections in the Immunocompetent Host

Usual Presentation:

The initial manifestations of IM include malaise, fatigue, feverishness, sweating, and chills and are due to the primary viremia and presumably to the production of interferon and interleukin-1. The sore throat, lymphadenopathy, and fever, which are the most characterisitc manifestations of IM, are probably secondary to the replication of the virus in the oropharynx, the infection of B lymphocytes, and the strong humoral and cellular immune responses. In some cases, tonsillar and pharyngeal edema may lead to pharyngeal obstruction. Mild to moderate headaches occur frequently during IM. They are usually retro-orbital and last for less than a week; however, in some cases, headaches may be severe, mimicking meningitis. There have been unconfirmed reports suggesting that EBV may play a role in cases of persistent fatigue (Straus *et al.*, 1985; Jones *et al.*, 1985). Although exacerbations of IM can occur, true recurrences of IM or reinfection with EBV are rare. Following IM, some patients complain of persistent fatigue and malaise, which may last for several months, and a variety of eponyms have been used to describe this syndrome, including postviral chronic fatigue syndrome (CFS), neurasthenia, epidemic neuromyasthenia, Iceland disease, and epidemic myalgic encephalitis. This postviral CFS may also occur after other viral infections, such as influenza, HHV-6, and enteroviral infections (Levine *et al.*, 1989). The majority of cases, however, have an uncertain etiology. In some instances the EBV-associated postviral CFS is not preceded by IM, and it may be very difficult to differentiate it from depression or from other infections such as enteroviral infections, lyme disease or even chronic brucellosis. Approximately 30% of muscle biopsies from patients with CFS contain enteroviral RNA or EBV

DNA (reviewed in Levine *et al.*, 1989), which does suggest an etiologic role for these viruses. The postviral CFS may be classified into epidemic and sporadic forms; EBV is a well documented cause of at least some of the sporadic forms of CFS (Tobi *et al.*, 1982; Alfieri *et al.*, 1984; Straus *et al.*, 1985). Patients with the EBV-associated chronic fatigue syndrome are usually identified by their abnormal immune response and high EA-IgG and VCA-IgG antibody titers (Levine *et al.*, 1989).

Atypical Presentation and Complications:

With the exception of headaches, symptoms related to the central nervous system are infrequent, but may be serious. EBV infections may present as meningitis, encephalomyelitis, polyneuritis, and mononeuritis. Signs and symptoms may vary from severe headaches, delirium, and acute psychosis to altered consciousness, coma, seizures, or paresis of the extremities. In most instances complete recovery from neurologic disease occurs (Chevenick, 1983). As noted earlier, infection by EBV results in a spectrum of immune responses, including a transient suppression of cell-mediated immunity and the production of a multiplicity of antibodies. In some instances there may be immunologic complications secondary to the EBV infection, such as autoimmune hemolytic anemia, acquired hypogammaglobulinemia, aplastic anemia, agranulocytosis, and immune complex formation, resulting in vasculitis, arthritis or nephritis (Bartley and Shulman, 1983). Sporadic fatal cases of IM do occur, but they are rare. Death usually results from neurologic complications, splenic rupture, superinfection secondary to leukopenia, or from hepatic necrosis with coagulopathy and hemorrhage (Weisenburger and Purtilo, 1986).

EBV Infections in the Immunocompromised Host

High titers of EBV-specific antibodies are frequently demonstrated in patients with sarcoidosis, systemic lupus erythematosus, rheumatoid arthritis, chronic kidney diseases, ataxia-telangiectasia, Hodgkin's disease, B-cell lymphomas, leukemias, carcinomas of the head and neck, and Kaposi's sarcoma. A common characteristic of these diseases is that they are immunodeficient conditions (Henle and Henle, 1979). The high titers of EBV-specific antibodies probably reflect an increased activation of the virus in immunodeficient states. In contrast, many patients with severe EBV infections are immunodeficient and do not exhibit the typical clinical or immune responses that are present in normal individuals. For instance, some immunodeficient patients do not develop heterophil antibodies or EBV-specific IgM antibodies after primary infection (Henle and Henle, 1979), and tests necessary for diagnosis, such as nucleic hybridization or lymphocyte transformation, are not generally available. Weisenburger and Purtilo (1986) compared the clinical manifestations of sporadic fatal IM and fatal IM in the X-linked lymphoproliferative syndrome (XLP) and found that the median age of patients with sporadic fatal IM was 13 years, while for patients with XLP it was only 2 years. The male to female ratio in sporadic cases was 1:1, while all patients with XLP were boys. Patients in both groups presented with typical symptoms and signs of IM. The disease was progressive, resulting in death within an average timespan of 4 weeks after onset. Patients with XLP have severe EBV infections, such as fatal or chronic IM, agamma or hypoglobulinemia, red cell aplasia, aplastic anemia, pseudolymphoma, and B cell malignant lymphoma. Purtilo *et al.* (1982) prospectively studied patients with XLP and were able to demonstrate some of the immune defects characteristic of this syndrome,

such as failure of the secondary immune response to the intravenous injection of bacteriophage ΦX174, which is characterized by the failure of switching from IgM to the IgG anti-ΦX174 antibody production. Lymphocytes from these patients also show decreased transformation when challenged with pokeweed mitogen (PWM) *in vitro*. Furthermore, the secretion of immunoglobulin into the culture medium is deficient. These findings suggest the presence of a defect in helper T cell regulation of B cells (Lindsten *et al.*, 1982). Patients with XLP fail to mount an anti-EBNA response and demonstrate persistently inverted T4:T8 ratios, hypogammaglobulinemia, and decreased NK cell activity (Sakamoto *et al.*, 1980).

Individuals who undergo iatrogenic immunosuppression, such as organ transplant recipients, and patients with congenitally acquired immunodeficiencies, such as ataxia-telangiectasia, and HIV-seropositive individuals are prone to developing active EBV infections and EBV-associated diseases such as hepatitis, myocarditis, encephalitis, bone marrow suppression, hemorrhage, pneumonitis, further immunosuppression resulting in opportunistic infections, and lymphoproliferative disorders. The clinical and histopathological features in immunosuppressed patients are virtually identical to those encountered in patients with fatal IM (Hanto *et al.*, 1984).

Organ transplantation is a frequent form of treatment that requires immunosuppressive therapy and puts patients at risk of developing opportunistic infections and *de novo* cancers. The risk of neoplastic disease is increased approximately 100-fold in immunosuppressed patients when compared with normal individuals, but it varies with the degree of immunosuppression, the

immunosuppressive regimen employed, and, in the case of organ transplant recipients, with the nature of the allograft. Except for skin cancers, the most common malignancies in this population are the lymphoreticular cancers. In contrast, cancers seen in the general population, such as lung, heart, and colon carcinomas, are rare among immunosuppressed patients (Penn, 1979). After renal transplantation, 1-13% of patients develop lymphoproliferative disorders (Frizzera, 1981). In addition, 13% of cardiac transplant recipients develop lymphomas (Bebier *et al.*, 1983). These data are from the earlier trials with cyclosporine. Rates are lower in recent studies. Cleary *et al.* (1986) have reviewed the evidence implicating EBV as the causative agent of lymphoproliferative disorders and transplant-associated lymphoreticular tumors. First, EBV is capable of infecting and immortalizing B lymphocytes to induce a polyclonal proliferation *in vitro* (Pope *et al.*, 1968). Second, EBV produces a self-limited lymphoproliferation in IM and experimental IM. EBV infection induces lymphoproliferation in cotton-top tamarins (Frank *et al.*, 1976). Third, in congenitally immunodeficient patients with the XLP syndrome, EBV is associated with fatal lymphoproliferative disease (Purtilo *et al.*, 1979). Fourth, immunosuppressed transplant recipients have an increased rate of oropharyngeal shedding of EBV (Chang *et al.*, 1978). In addition, most of the lymphoreticular tumors in renal and cardiac transplant recipients contain EBV DNA (Hanto *et al.*, 1981; Saedmundsen *et al.*, 1982). Similarly, EBNA and EBV DNA are also found in the tumor tissues of recipients of heart-lung, liver, bone marrow, and thymic epithelial transplants who develop lymphomas (Cleary *et al.*, 1986). Immunosuppressed children who develop a primary infection with EBV frequently develop an aggressive form of lymphoproliferative disorder (Ho *et al.*, 1985). The above evidence supports the fact that EBV is at least in part

responsible for the lymphoproliferative disorders found in immunosuppressed patients.

Controversy exists as to whether the B cell lymphoproliferation, which is initially polyclonal and may be driven by EBV, is the first step in the development of these malignancies. The process, which is thought to be initially polyclonal, may become oligoclonal or monoclonal with a chromosomal translocation. The behavior of these tumors does not always correlate with clonality as defined by conventional techniques. Patients have been described who had fatal disease despite the polyclonal nature of the lymphoproliferative tissue as defined by surface immunoglobulin markers; however, techniques such as analysis of the rearrangement of the immunoglobulin gene and characterization of the clonotypic characteristics of the EBV genome found in the tumor may reveal that, in fact, these tumors are oligoclonal or monoclonal. Therapy of the lymphoproliferative disorders has consisted of decreasing the immunosuppressive agents and administration of antiviral agents such as acyclovir or gancyclovir. However, the results of such therapies have been discouraging, and the mortality rate of the lymphoproliferative disorders continues to be high (Schooley, 1987).

Antiviral Agents Active Against EBV

EBV has two life cycles, a latent and a lytic growth cycle. Each of these stages of replication is carried out by different enzymatic systems. During latency the cellular DNA polymerase replicates the episomal forms of the EBV genome, but cellular enzymes are not susceptible to antiviral agents (Sixbey and Pagano,

1985). In contrast, during the lytic growth cycle, linear molecules of the viral genome are replicated by the viral DNA polymerase which may be inhibited by antiviral agents (Katz *et al.*, 1989).

Although antiviral agents do not affect EBV infection once latency is established, lymphocytes exposed to interferon (IFN)-α before infection do not become transformed and immortalization by EBV may be prevented (Lin *et al.*, 1989). These observations may have implications for the treatment of lymphoproliferative disorders induced by EBV. Shapiro *et al.* (1988) successfully treated five organ transplant recipients having B cell lymphoproliferative disorders with a combination of IFN-α and IV gamma globulin. The mechanism of action of IFN-α is uncertain, although it may act through a direct antiviral effect, an antiproliferative effect on B lymphocytes, or by enhancing the cellular immune response to infected cells.

Several nucleoside analogs selectively inhibit the replication of EBV during the lytic growth cycle. Acycloguanosine (ACV) and dehydroxyacycloguanosine (DHPG) have potent effects against EBV replication *in vitro* (Lin *et al.*, 1984). Other nucleoside analogs, such as E-5-(2-bromovinyl)-2'-deoxyuridine, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine, and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil have antiviral effects on EBV *in vitro* (Lin *et al.*, 1983).

ACV induces regression of oral hairy leukoplakia, an epithelial lesion caused by EBV (Resnick *et al.*, 1988). ACV can also inhibit the oropharyngeal shedding of EBV and decrease the duration of fever, weight loss, tonsillar swelling, and

pharyngitis during infectious mononucleosis (Andersson *et al.*, 1986), but it does not have an effect on the number of B cells latently infected by EBV in the peripheral circulation (Yao *et al.*, 1989a, 1989b).

AZT, which inhibits the replication of HIV, also suppresses EBV *in vitro*. The combination of acyclovir and AZT has an additive effect against EBV replication (Lin *et al.*, 1988). It is uncertain whether AZT has any effect on EBV *in vivo*, since leukoplakia does not regress with AZT therapy alone (Resnick *et al.*, 1988).

Although there are no specific indications for antiviral therapy during EBV infections, the preliminary results of the combined administration of IFN-α and gamma globulin in LPD should promote further clinical trials with these and other antiviral agents in the therapy of EBV-associated lymphoproliferative disorders.

STATEMENT OF THE PROBLEM AND THE APPROACH

The purpose of this thesis project was to develop DNA probes for the detection of EBV DNA in clinical specimens, to validate their use, and to apply the technique to some relevant clinical problems.

When the work described in this thesis was started, the EBV genome was already cloned and sequenced, but to my knowledge, there were no studies validating the clinical use of EBV DNA probes. We initially compared the accuracy, sensitivity, and specificity of a dot-blot hybridization assay with the classical diagnostic tests for detection of EBV infection, such as lymphocyte transformation and EBV-specific serology. Most clinical studies examining the role of EBV in conditions such as the "chronic fatigue syndrome," fibrositis, persistent headaches, lymphoproliferative disorders in immunosuppressed patients, and EBV infections in HIV-seropositive individuals, were limited in their analysis to EBV-specific serology and, in a few cases, attempted to culture the virus from throat washings. However, there is evidence that the EBV-specific serology and the lymphocyte transformation are not ideal methods for studying these clinical problems. For instance, immunocompromised patients, such as organ transplant recipients, and HIV-seropositive individuals may have deficiency of the T helper function, resulting in an altered humoral immune response to EBV (Blumberg *et al.*, 1987) which may not reflect the true state of an EBV infection (latent or active). EBV-specific serology could be a poor indicator of EBV activation in immunocompromised individuals. Similarly, the lymphocyte transformation assay had intrinsic problems which have already been discussed. I wanted to apply the

DNA probe technology to some of these ill-defined clinical syndromes in which EBV has been hypothesized to play a role.

To predict whether individuals would respond to antiviral therapy, it was necessary to define the molecular configuration of the EBV genome detected by DNA probes in oropharyngeal cells. Only linear forms are susceptible to the inhibitory action of nucleoside analogs such as acycloguanosine (acyclovir) or dihydroacycloguanosine (gancyclovir). In order to define whether linear forms of EBV were predominant in oropharyngeal cells, the telomeres of intracellular EBV genomes were analyzed by Southern blot analysis of DNA from oropharyngeal cells. This will help define replicating from episomal forms of DNA and may be useful in predicting susceptibilities to antiviral therapy.

I was also interested in the effects of EBV activation and levels of EBV excretion on HIV infection and whether high levels of EBV excretion could predict or be a co-factor in the progression of HIV disease. Although the immunodeficiency observed in HIV infection could be the sole cause of EBV activation, EBV has the potential to increase the immunosuppression and to activate the replication of HIV in cells infected by both viruses. In addition, one of the immediate early antigens of EBV may serve as transactivator for HIV replication (Kenney *et al.*, 1987).

EBV infections were also studied during episodes of severe immunosuppression in organ transplant recipients. The hypothesis was that activation of EBV in oropharyngeal cells would be a good indicator of the degree of immunosuppression. The levels of EBV excretion in oropharyngeal cells could predict the emergence of, and therefore identify patients at risk for,

lymphoproliferative disorders or superinfections. Another hypothesis is that patients undergoing organ transplantation are susceptible to reactivation, reinfection or primary infection with a transforming strain of EBV, which could be associated with aggressive lymphoproliferation.

In summary, the unifying hypothesis of this thesis correlates the derangement of the cellular immune response with the increased replication of EBV in oropharyngeal cells and the biologic effects of an increased virus load.

Table I-1

Spectrum of EBV-Associated Diseases

Conditions in Which EBV Plays an Etiologic Role	Some of the Conditions in Which EBV Has Been Associated but in Which its Etiological Role is Unclear
1. Acute, recurrent, or chronic infectious mononucleosis	1. Lymphoid pneumonitis in children with AIDS
2. Lymphoproliferative disorders in immunocompromised individuals	2. Thymic carcinoma
3. Hairy leukoplakia	3. Hodgkin's disease
4. African Burkitt's lymphoma	4. Colonic lymphoid hyperphasia in HIV-seropositive individuals
5. Nasopharyngeal carcinoma	5. Midline granuloma
	6. Chronic fatigue syndrome
	7. Persistent headaches

Table I-2

Humoral Antibody Responses in Patients With Infectious Mononucleosis

Major Antibodies	Minor Antibodies
1. Heterophil-IgM (Paul-Bunnell-Davidsohn)	1. Rheumatoid factor
2. Antibodies to Epstein-Barr virus antigens	2. Antinuclear
3. Cold agglutinins (anti-i)	3. Antimitochondrial
4. Antibodies to smooth muscle	4. Antireticulin
	5. Antimicrosomal
	6. Anti-intermediate filaments
	7. Lymphocytotoxins
	8. Wassermann reagin

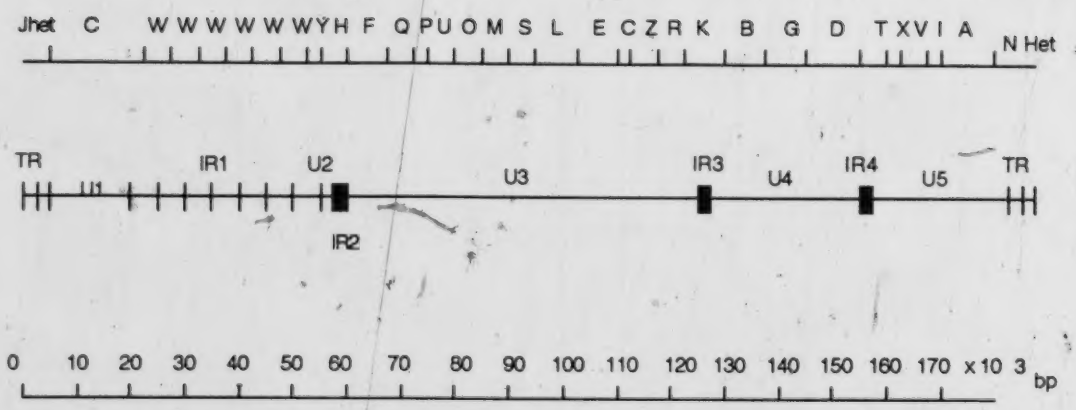
Jhet C W W W W W W Y H F Q P U O M S L E C Z R K B G D T X V I A N Het
TR
IR1
U2
U3
IR3
U4
IR4
U5
TR
IR2
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 x 10 3 bp

Figure I-2. Bam HI restriction map and genomic organization of the EBV

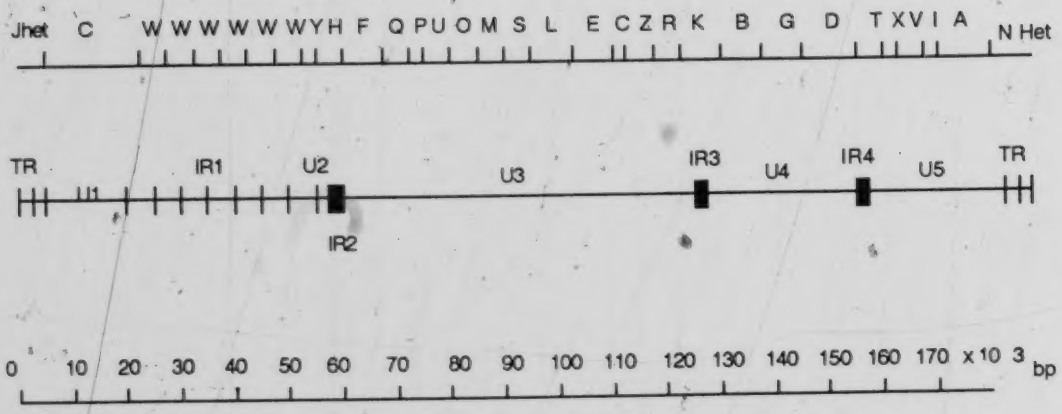
Jhet C W W W W W W Y H F Q P U O M S L E C Z R K B G D T X V I A N Het
TR U1 IR1 U2 IR2 U3 IR3 U4 IR4 U5 TR
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 x 10 3 bp

Figure I-2. Bam HI restriction map and genomic organization of the EBV

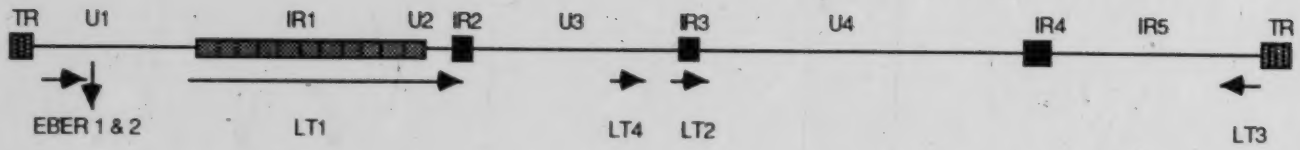

Figure I-3. Regions of the Epstein-Barr virus transcribed during latency. The EBV-encoded small RNAs, termed EBERs, are 166 an 177 nucleotides long, they are transcribed by RNA polymerase III. Their biochemical or biologic role is not known. LT1 encodes for the EBNA-2 and its leader protein. LT2 encodes for EBNA-1, which is important in the maintenance of the episomal state. LT3 encodes for the latent membrane protein (LMP), which may have a a role in cellular activation. LT4 encodes for the EBNA-3 gene product. The region adjacent to the left telomere encodes the small non-polyadenylated RNAs.

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CHAPTER II

VALIDATION OF THE DNA-DNA DOT HYBRIDIZATION TO DETECT EPSTEIN-BARR VIRUS IN THROAT WASHINGS

A VERSION OF THIS CHAPTER HAS BEEN PUBLISHED as, "DNA-DNA dot hybridization to detect Epstein-Barr virus in throat washings." F. Diaz-Mitoma, J.K. Preiksaitis, W.-C. Leung, D.L.J. Tyrrell. *J. Infect. Dis.* **155**: 297-303, 1987.

Introduction

The laboratory diagnosis of EBV infections depends on serology or on detection of the virus, viral antigens, or viral DNA. Confirmation of the diagnosis of acute infectious mononucleosis usually depends on detecting heterophil antibodies. EBV-specific antibodies are usually detected by an indirect immunofluorescence assay. The status of an EBV infection can be defined by demonstrating an antibody response to virus-specific antigens. IgM antibodies to viral capsid antigen (VCA) are produced in response to acute EBV infection. Persistently elevated titers of antibody to the early antigens are thought to be evidence of ongoing viral infection. The presence of IgG antibodies to VCA and EBV-associated nuclear antigens (EBNA) indicates past infection (Henle *et al.*, 1974). Biologically active virus can be demonstrated in throat washings (pharyngeal epithelial cells), circulating B lymphocytes, or lymphoid tissue by transformation of lymphocytes from umbilical cord blood. This assay is cumbersome, time-consuming (three to eight weeks), and requires specialized tissue culture facilities that are not available in many routine diagnostic laboratories. The isolation of EBV is therefore not frequently attempted, and serological tests are often used for laboratory confirmation of EBV infections.

Nucleic acid hybridization represents a sensitive and specific method of demonstrating virus-specific DNA in clinical specimens (Brandsma and Miller, 1980; Kawai *et al.*, 1973). The development of cloned subgenomic fragments of

EBV has made new reagents available for the detection of EBV in clinical specimens (Pi *et al.*, 1983; Fischer *et al.*, 1981; Andiman *et al.*, 1983).

In this report we present our experience on the use of DNA-DNA hybridization technology to identify EBV in cells from throat washings. The purpose of this study was to validate the use of a DNA probe for detecting EBV DNA in exfoliated oropharyngeal cells and to compare the results of dot hybridization to those of cord lymphocyte transformation in renal transplant recipients, patients with acute infectious mononucleosis, and healthy EBV-seropositive and EBV-seronegative controls.

Materials and Methods

Population:

Patients with Acute Infectious Mononucleosis: Throat washing specimens were collected from eight patients during the first two weeks of an illness compatible with the diagnosis of acute infectious mononucleosis. At the time the specimens were collected, all patients had severe pharyngitis, a positive Monospot test of IgM antibodies to VCA, and >15% atypical lymphocytes on a smear of peripheral blood.

Renal transplant recipients: Thirty-eight specimens from 26 renal transplant recipients were tested for EBV by dot hybridization and lymphocyte transformation. Thirteen specimens were taken before transplantation and 25 were taken afterwards. All renal transplant recipients received cyclosporin A and prednisone.

Controls: The control group of 60 laboratory and medical personnel included 9 EBV-seronegative subjects and 51 healthy EBV-seropositive individuals. The samples from patients and controls were randomly assigned coded numbers before the dot hybridization and lymphocyte transformation tests.

Patients and control subjects gargled with 10 ml of RPMI 1640 medium containing penicillin and gentamicin. After patients had gargled with the medium, fetal calf serum was added to the medium to a final concentration of 10%. Epithelial cells in the throat washings were pelleted at 800 *g* for 10 min, and the supernatant was stored at -70°C. Supernatants from controls and from patients with acute infectious mononucleosis were stored for an average of 10.5 months until assayed by lymphocyte transformation. Some pellets were resuspended before freezing to examine the nature of cells contained in throat washings. All cells were of epithelial origin.

The cell pellets were lysed as previously described (Meinkoth and Wahl, 1984). Briefly, the cell pellet was incubated with 0.5% SDS and 200 μg of proteinase K/ml for 1 hr at 37°C. This incubation was followed by extraction with 1 volume of phenol:chloroform (vol/vol) and 1 volume of chloroform. After adding 1/10 volume of 3 *M* sodium acetate (pH 5.2), the DNA was precipitated with ethanol. The DNA pellet was resuspended in *tris*-EDTA [0.01 *M tris*-HCl (pH 7.0)] and 0.001 *M* EDTA] buffer and boiled for 5 min to denature the DNA. The DNA suspensions were immediately chilled on ice and neutralized by adding an equal volume of 2 *M* NH_4COOCH_3 (pH 7.0). The DNA samples were spotted onto nitrocellulose filters (BA85; Schleicher & Schuell, Keene, N.H.) with a manifold filter (Bio-Rad, Richmond, Calif.). The filters were air-dried and baked for 1 hr at 80°C. The filters were then incubated for 1 hr at 64°C in 6 x SSC (1 x SSC = 0.15 *M* NaCl and 0.15 *M* sodium citrate), and 5 x Denhardt's solution supplemented

with 100 µg of sheared and denatured salmon DNA/ml. The hybridization was carried out in a solution of 6 x SSC and 5 x Denhardt's solution with 0.5% SDS containing 10^6 cpm/ml of EBV DNA probe (the probe had been denatured by boiling for 5 min in 0.3 *M* NaOH). The filters were incubated for 24 hr at 64°C for hybridization. Post-hybridization washings were carried out twice in 2 x SSC with 0.1% SDS at room temperature for 5 min each, and in 0.2 x SSC with 0.1% SDS at 64°C for 1 hr (Meinkoth and Wahl, 1984). The radioactivity bound to the DNA dots was detected by autoradiography by exposure for 12-24 hr at -70°C with X-ray film (XAR-5 film, Eastman-Kodak, Rochester, N.Y.) and by counting individual dots in a "1215 Rackbeta" liquid scintillation counter (Wallac, Turku, Finland).

A positive dot could be detected visually by autoradiography and confirmed quantitatively by cutting out the filters to count radioactivity. A positive dot had a radioactivity count of more than two standard deviations above the mean cpm of the control population. DNA samples extracted from cytomegalovirus, human umbilical cord lymphocytes, and placental tissue were used as negative controls in all filters. DNA samples from the recombinant plasmid or the cell line B95-8 (marmoset lymphocytes carrying EBV) were used as positive controls in all filters.

DNA Probe:

The EBV probe was obtained from an EBV genomic DNA library kindly provided by Dr. B. Sugden (University of Wisconsin, Madison, Wis.). The recombinant plasmid was pBR325 containing the *Bam*HI-W fragment of the EBV genome. The plasmid was propagated in *Escherichia coli* HB101 and purified by equilibrium centrifugation in a cesium chloride-ethidium bromide gradient (Maniatis *et al.*, 1982). The probes consisted of the recombinant plasmid containing the *Bam*HI-W fragment or the purified *Bam*HI-W fragment excised

from the plasmid, which is the IR1 and 3072 bp in length (Figure I-2). The *Bam*HI-W subgenomic fragment was chosen because repeated sequences may amplify the sensitivity of the assay. To determine the sensitivity of the dot assay, we labeled the probes with (^{32}P)-dCTP (by nick translation) to a specific activity of 10^8 cpm/μg of DNA (Rigby *et al.*, 1977). Titration experiments were done by using 1 x 10^6 cpm of radiolabeled probe and tenfold dilutions of DNA extracted from the recombinant plasmid, B95-8, or Raji cells. No differences could be detected in the results of dot hybridization when either the recombinant plasmid or the purified *Bam*HI-W fragment were used as probes.

Lymphocyte Transformation:

Throat-washing supernatants were filtered (0.45-μm filter), and the filtrate was tested for transforming activity in umbilical cord lymphocyte cultures as described (Niederman *et al.*, 1976). The supernatant (1 ml) was inoculated onto 2 x 10^6 cord lymphocytes in 1.5 ml of RPMI 1640 containing 10% fetal calf serum. The presence of EBV was demonstrated by transformation of the lymphocytes. The presence of EBV in transformed lymphocytes was confirmed by dot hybridization.

Serology:

Serum samples were obtained when the throat washing specimens were collected from patients and controls. Titers of IgG antibody to VCA and to early antigens were measured by the indirect immunofluorescence test as previously described (Henle *et al.*, 1974; Ito *et al.*, 1981). The determination of antibodies to the early antigens did not distinguish between diffuse and restricted components.

Results

The sensitivity of the dot hybridization assay was determined by spotting the DNA from a known number of B95-8 and Raji cells on filters. The Raji cell line contains ~60 EBV genomes per cell (Adams, 1979). The radioactive probe could detect 5-10 pg of homologous DNA (Figure II-1, Lane B), 100 Raji cells, or 10 B95-8 cells (~6000 EBV genomes) per spot (Figure II-1, Lane C). Dots containing DNA extracted from oropharyngeal cells from patients with acute mononucleosis and from renal transplant recipients are also shown in Figure II-1 (Lanes A, D, E, and F).

The dot hybridization assay appeared to be specific, as there was no cross-hybridization with human DNA from umbilical cord lymphocytes, placental tissue, or cytomegalovirus DNA (Figure II-1, Lane G). The highly stringent conditions used for the post-hybridization washings eliminated most of the nonspecific background radioactivity. Nonspecific reactions with the negative cell controls could not be demonstrated. Positive dot hybridization (as determined by visual examination of the autoradiograms) correlated well with the results obtained by radioactivity counts.

The mean cpm of the dot hybridization for the controls was significantly lower ($p < 0.001$) than the mean cpm for patients with acute mononucleosis or renal transplant recipients (Figure II-2).

The results of the dot hybridization and lymphocyte transformation assays performed on 106 specimens are summarized in Table II-1. None of the 9 EBV-

seronegative controls had EBV detected by the lymphocyte transformation or the dot hybridization assays. Among the 51 EBV-seropositive controls, there were 4 subjects who had a positive dot hybridization test (dot-positive). Three of these 4 subjects had EBV detected by the lymphocyte transformation assay (transformation-positive). One control subject had throat washings which were dot-negative and transformation-positive. None of the control subjects who had dot-positive or transformation-positive specimens reported any symptoms at the time of collection of throat washings, nor did they recall ever having had infectious mononucleosis.

There was complete correlation between the dot hybridization and lymphocyte transformation assays in patients with acute infectious mononucleosis. Throat washings obtained from 7 (87.5%) of 8 patients with proven infectious mononucleosis were positive on both dot hybridization and lymphocyte transformation. The patient whose throat washings were negative by both assays was admitted to the hospital with classical infectious mononucleosis. She demonstrated extensive cervical lymphadenopathy and had a high fever. The titer of IgM antibody to VCA was 1:8 in this patient.

Thirty-eight specimens collected from 26 renal transplant recipients were tested for EBV by both tests. Fourteen were positive by dot hybridization and 10 by lymphocyte transformation. Only 7 were concordant. The low rate of concordance in specimens from renal transplant recipients may have been the result of longer periods of storage at -70°C before the lymphocyte transformation assay was performed. Specimens from controls and from patients with acute mononucleosis were stored for a mean of 2.1 months, and specimens from the

renal transplant recipients were stored for a mean of 10.5 months before the lymphocyte transformation assay. There were 12 (11%) discordant results between the lymphocyte transformation and dot hybridization assays in the 106 specimens tested (Table II-1). Eight of these 12 specimens were hybridization-positive and transformation-negative. These 8 specimens were stored at -70°C for an average of 11 months (renal transplant recipients) before lymphocyte transformation, whereas the 17 specimens with positive results for both tests were stored for an average time of four months (range: 0-13 months) before lymphocyte transformation. Overall, the sensitivity and specificity of the dot hybridization assay were 81% and 90%, respectively. The sensitivity and specificity increased to 90% and 98% when the specimens from renal transplant recipients were excluded from the analysis.

Seven of the 51 seropositive controls had titers to early antigen >1:10. Four of these patients were excreting EBV, but only 1 of the 44 early antigen-negative patients excreted virus. For comparison, titers of VCA and early antigen were determined for 24 renal transplant recipients. All were seropositive and had titers of VCA >1:20. The titer of early antigen was >1:10 in 15 patients, of whom 7 excreted EBV and 8 did not. A total of 13 renal transplant recipients excreted EBV.

Discussion

Several studies have shown that oropharyngeal epithelial cells are a site of EBV replication in humans (Lemon *et al.*, 1977; Sixbey *et al.*, 1984). DNA probes

containing the *Bam*HI-W repeating subgenomic fragment of EBV have been used to detect EBV in lymphoma tissue (Andiman *et al.*, 1983), bronchial cells (Lung *et al.*, 1985), and exfoliated epithelial cells in patients with nasopharyngeal carcinoma (Pi *et al.*, 1983). Our study demonstrates the high sensitivity and specificity as well as some of the clinical applications of the dot hybridization assay for detecting EBV in exfoliated oropharyngeal epithelial cells. When this assay was compared with the transformation assay, discordant results were obtained in 12 (11%) of 106 specimens analyzed. Eight of these specimens were dot-positive and transformation-negative. These results might be explained by inactivation of the transforming ability of EBV because of the prolonged storage of frozen specimens. Support for this explanation was found when the storage time for dot-positive, transformation-positive specimens was compared with the storage time for dot-positive, transformation-negative specimens. Specimens that were stored for less than four months were more likely to be concordant by both tests. Specimens stored for more than four months gave a higher proportion of dot-positive, transformation-negative results. One explanation is that oropharyngeal secretions of EBV-seropositive patients may contain an antibody that interferes with transformation. Another possible explanation is that transformation may be defective in some strains of EBV. The isolation of nontransforming strains has been described in patients with chronic mononucleosis (Alfieri *et al.*, 1984); however, the frequency of nontransforming virus in EBV infections has not been studied. The four hybridization-negative, transformation-positive results suggest that transformation was more sensitive than hybridization in these specimens. Ideally, transformation assays should be done immediately, but logistically this was not possible since fresh cord lymphocytes are required for the assay.

Ten percent of the asymptomatic seropositive control subjects demonstrated oropharyngeal excretion of EBV without having a recent history of infectious mononucleosis. Other researchers (Gerber *et al.*, 1972; Miller *et al.*, 1973) have reported a similar prevalence of asymptomatic excretion of EBV in normal adults. The presence of EBV in the oropharynx of healthy seropositive individuals is thought to represent reactivation of latent EBV.

This study describes several clinical settings in which detection of EBV may be useful. The role of EBV in patients with CFS is uncertain. Patients have nonspecific symptoms of headache, fatigue, sore throat, and paresthesias. The symptoms are common, and the detection of an active EBV infection could help to define the role that EBV may play in this syndrome. The detection of EBV would be particularly important if such patients are to receive antiviral therapy for CFS. These studies would require double-blind placebo-controlled trials with monitoring for clinical and virological responses. The dot-blot hybridization assay may be very useful in such studies.

Patients who are immunosuppressed or receiving chemotherapy may have altered serological responses to EBV. Bone marrow transplant recipients who were seronegative for antibodies to EBV failed to develop IgM antibody responses to VCA when infected with EBV transmitted by the donor marrow (Henle and Henle, 1981). Renal allograft recipients may not demonstrate heterophil antibodies during primary EBV infection. Unlike normal subjects, only 50% of allograft recipients develop IgM antibodies to VCA (Ho *et al.*, 1985). Serological responses in patients receiving transfusions may also be difficult to

interpret because of passive transfer of antibodies to EBV. Ho *et al.* (1985) found that only 16% of allograft recipients treated with cyclosporine had serological evidence of active EBV infection. In contrast, we found that 50% of renal allograft recipients receiving cyclosporine demonstrated EBV oropharyngeal shedding as measured by dot-blot hybridization and lymphocyte transformation assays. Serology was not a good method of identifying infection in these patients. Other studies have shown (by using the cord lymphocyte transformation assay) that 47-83% of renal allograft recipients undergoing other immunosuppressive regimens shed EBV from oropharyngeal cells (Strauch *et al.*, 1974; Chatterjee and Chang, 1982). The dot hybridization assay provides a simple and rapid method for detecting EBV shedding and allows for longitudinal follow-up of EBV excretion in allograft recipients. Lymphoproliferative disorders in allograft recipients are a well-recognized complication of transplantation. There is increasing evidence for the association of these disorders with EBV infection (Ho *et al.*, 1985). Patients at high risk of lymphoproliferative disorders might be identifiable by an unusually long duration or high titer of excreted EBV.

The dot hybridization assay for EBV is a sensitive and specific test that can be performed on oropharyngeal cells. For specimens stored for long periods (longitudinal studies), the dot hybridization assay may be more sensitive than the lymphocyte transformation assay. It is rapid and can be used to screen large numbers of clinical specimens. This study demonstrates that dot hybridization is an additional tool for the study of the natural history of EBV infection in a number of clinical settings.

Table II-1

Comparison of the Dot-Blot Hybridization and Lymphocyte Transformation Assays in Detecting EBV Genome in Throat Washings From Controls, Patients With Acute Infectious Mononucleosis, and Renal Transplant Recipients

Patients, results of dot blot hybridization assay	Results of lymphocyte transformation assay		
	Positive	Negative	Total
Controls			
EBV-seronegative (n = 9)			
Positive	0	0	0
Negative	0	9	9
EBV-seropositive (n = 51)			
Positive	3	1	4
Negative	1	46	47
Patients with acute infectious mononucleosis (n = 8)			
Positive	7	0	7
Negative	0	1	1
Renal transplant recipients (n = 38)			
Positive	7	7	14
Negative	3	21	24

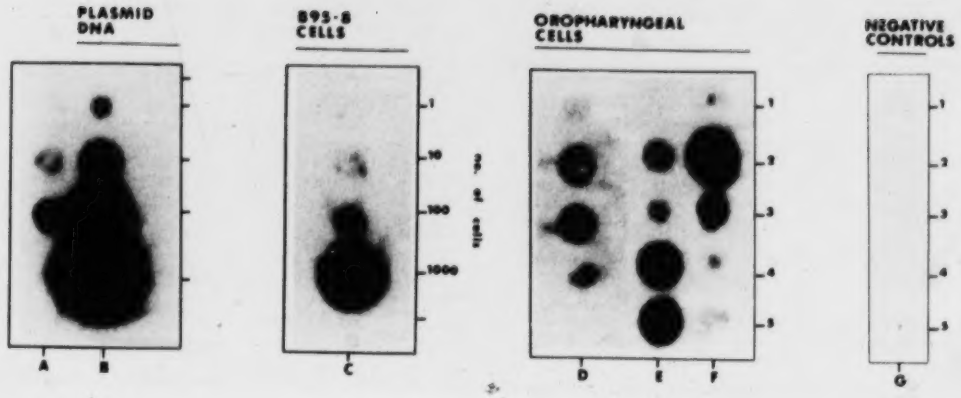

Figure II-1. Nucleic acid dot hybridization with the purified ^{32}P-labeled *Bam*H1-W subgenomic fragment of EBV. *(A)* DNA extracted from oropharyngeal cells of two renal transplant recipients who were excreting EBV; weakly positive *(upper)* and strongly EBV-positive *(lower)*. *(B)* Titration of the recombinant plasmid DNA. The plasmid was spotted in serial 10-fold decreasing dilutions starting at 10 ng. *(C)* DNA extracts from B95-8 cells were spotted in serial 10-fold decreasing dilutions starting with the DNA extracted from 10^3 cells per dot. *(D)* DNA extracted from oropharyngeal cells. Dots *D1* and *D5* are from EBV-seronegative subjects; *D2* (400,000 cells), *D3* (400,000 cells), and *D4* (350,000 cells) are from patients with acute infectious mononucleosis. *(E, F)* DNA extracted from oropharyngeal cells of renal transplant recipients. Dots *E1* and *F5* are EBV-negative specimens; *E3*, *F1*, and *F4* are weakly positive; and *E2*, *E4*, *E5*, *F2*, and *F3* are strongly EBV-positive specimens. *(G)* Negative controls. The dot *G1* is the DNA extracted from 1 million fetal cord lymphocytes. Placental human DNA was spotted in *G2* (5 μg) and *G3* (1 μg). DNA extracted from fibroblasts infected with CMV was spotted on *G4* (5 μg) and *G5* (1 μg).

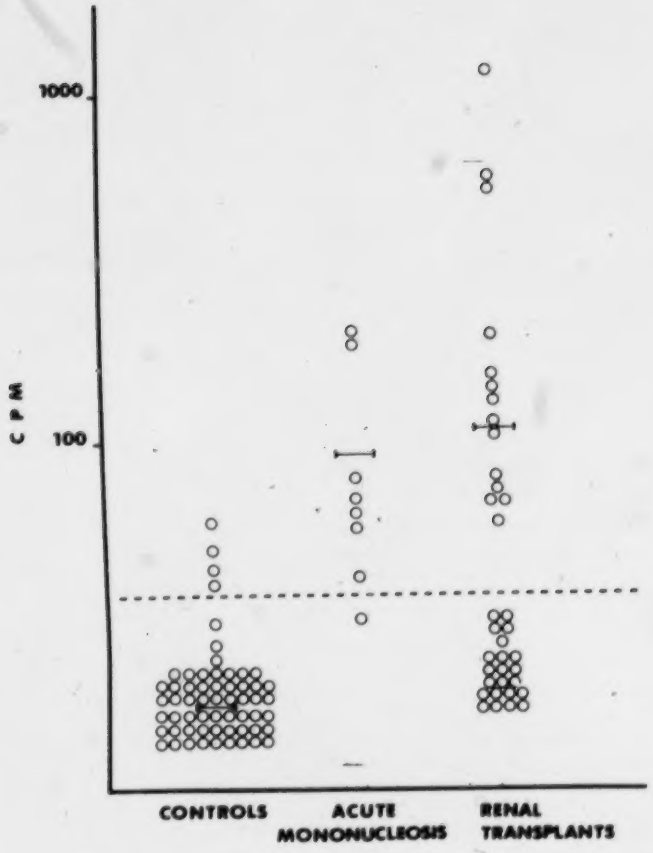

Figure II-2. Radioactivity counts from the dot hybridization assay for exfoliated oropharyngeal cells obtained in throat washings from 60 healthy controls (mean cpm: 17.4 ±9.2), 8 patients with acute infectious mononucleosis (mean cpm: 91.8 ±62.0; $p < 0.001$), and 38 specimens from 26 renal transplant recipients (mean cpm: 111 ±216; $p < 0.001$). Closed circles represent repeated specimens. Two standard deviations above the mean for controls was used as a break point (------) between positive and negative dot-blot hybridization. *P* values were calculated by comparing the mean cpm (± SD) of the dot hybridization in the control population with the mean cpm in patients with acute infectious mononucleosis or in renal transplant recipients. Horizontal bars = mean cpm.

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CHAPTER III

DIAGNOSTIC APPLICATIONS OF THE DNA PROBES: INCREASED FREQUENCY OF EPSTEIN-BARR VIRUS EXCRETION IN PATIENTS WITH NEW DAILY PERSISTENT HEADACHES

A VERSION OF THIS CHAPTER HAS BEEN PUBLISHED as, "Increased frequency of Epstein-Barr virus excretion in patients with new daily persistent headaches." F. Diaz-Mitoma, W.J. Vanast, D.L.J. Tyrrell. *Lancet* 1:411-416, 1987.

Introduction

Several reports have suggested that EBV may be associated with a chronic syndrome characterized by fatigue, headaches, paresthesias, and other non-specific symptoms (Isaacs, 1948; Chang and Maddock, 1980; Tobi *et al.*, 1982; Jones *et al.*, 1985; Straus *et al.*, 1985; Borysiewicz *et al.*, 1986). These studies have demonstrated that some patients with the chronic fatigue syndrome (CFS) have abnormally elevated titers of antibodies to the viral capsid antigen (VCA) or early antigen (EA). Many of these patients do not have antibodies to EBV nuclear antigen (EBNA) as detected by anticomplement immunofluorescence. DNA hybridization is a sensitive and specific method for the detection of EBV in oropharyngeal cells during EBV reactivation in symptom-free individuals, in patients with acute infectious mononucleosis, and in immunosuppresed patients (Diaz-Mitoma *et al.*, 1987).

Sixty to 78% of women in the general population have headaches (Waters and O'Connor, 1971; Paulin *et al.*, 1985). In one study, 65% of women with headaches regarded their headaches as severe or disabling (Sachs *et al.*, 1985). Idiopathic chronic headaches are commonly classified as migraine or muscle contraction headaches (Ad Hoc Committee on Classification of Headaches, 1962). However, several reports have suggested that migraine and muscle contraction headaches represent a continuum rather than distinct clinical entities (Bakal and Kaganov, 1977; Tfelt-Hansen *et al.*, 1981; Featherstone, 1985). When such headaches occur daily the syndrome is referred to as "daily chronic

headache" (DCH) (Saper *et al.*, 1983; Saper, 1986). In the present study we have examined a subset of DCH which has been recently defined as "new daily persistent headache" (NDPH) (Vanast, 1986). This syndrome occurs primarily in young adults with no known headache precipitants such as trauma or psychological stress. The headaches are of sudden onset and usually, but not always, resolve spontaneously within a year. We suspected an association between NDPH and EBV because a high frequency of abnormally high EBV antibody titers and occasional positive Monospot tests have been found in patients with NDPH referred for neurological evaluation.

In this investigation we determined the frequency of EBV reactivation by means of serological and virus excretion studies in patients with NDPH.

Materials and Methods

Subjects:

Thirty-two NDPH patients (age 15 years or more) who were referred to the neurological service of the Edmonton General Hospital during an 18-month period took part in the study. At the initial evaluation all patients had headache which had persisted for at least one month. None had a past history of endocrine, autoimmune, or neoplastic disorders, and none had evidence of intracranial lesions or other neurological disorder as determined by history, physical examination, and computerized axial tomography. No patient was on immunosuppressant agents.

Controls matched for sex and age (±5 years) were recruited from healthy, symptom-free volunteers.

Specimen Collection and DNA Hybridization:

Throat washings for EBV DNA detection (obtained by asking the subjects to gargle with 20 ml normal saline for 15-20 s) were collected from all patients and controls at the initial evaluation and from 17 patients every 4 to 8 weeks during follow-up. The number of oropharyngeal cells obtained was increased by swabbing the throat with cotton-tipped swabs before gargling. The throat washings were centrifuged at 4000 *g* for 10 min, the supernatant was discarded, and DNA was extracted from the cellular pellet as described elsewhere (Meinkoth and Wahl, 1984). The cell pellet was incubated with 0.5% sodium dodecyl suphate (SDS) and 200 µg/ml of proteinase K for 1 hr at 37°C in a total volume of 200 µl. The incubation was followed by extractions with 1 volume phenol:chloroform (vol/vol), then with 1 volume chloroform. After the addition of 1/10 volume of 3 *M* sodium acetate (pH 5.2) and 10 mg tRNA as carrier, the DNA was precipitated with ethanol. The DNA pellet was resuspended in 0.3 *M* NaOH and incubated at 60°C for 5 min to denature the DNA. The DNA suspensions were immediately chilled on ice and neutralized with an equal volume of 2 *M* ammonium acetate (pH 7.0). The DNA samples were spotted onto nylon filters (Hybond-N, Amersham, Oakville, Ontario) with a manifold filter (Bio-Rad, Richmond, Calif.). The filters were air-dried, exposed to ultraviolet light for 2 min, then incubated for 2 hr at 64°C in 6 x SSC (1 x SSC = 0.15 mol/l NaCl, 0.015 mol/l sodium citrate) and 5 x Denhardt's solution supplemented with 200 µg/ml of sheared and denatured salmon DNA. For hybridization, the filters were incubated for 24 hr at 64°C in a solution of 6 x SSC and 5 x Denhardt's solution

plus 0.5% SDS containing 1 million cpm/ml of EBV DNA probe which had been denatured by boiling for 5 min. The filters were then washed in 2 x SSC at room temperature for 1 h and in 0.2 x SSC plus 0.1% SDS at 64°C for 30 min. Positive dots were detected by means of autoradiography exposure for 12-24 hr at -70°C with X-ray film (XAR-5 film, Kodak, Rochester, N.Y.).

DNA samples extracted from cytomegalovirus-infected cells, human umbilical cord lymphocytes, and placental tissue were used as negative controls in all filters. DNA samples from the recombinant plasmid, Raji cells, or the cell line B95-8 (marmoset lymphocytes carrying EBV) were used as positive controls in all filters.

DNA Probe:

The probe consisted of the purified *Bam*HI-W fragment excised from the recombinant plasmid as described in Chapter II. The probe was labeled with ^{32}P-deoxycytidine triphosphate by nick translation to a specific activity of $>10^8$ cpm/μg DNA (Rigby *et al.*, 1977).

Serology:

Serum samples were collected at the same time as the throat washings. IgG antibody titers to VCA and EA, and IgM antibodies to VCA were measured with the indirect immunofluorescence test (Henle *et al.*, 1974; Ito *et al.*, 1981). Antibody determination did not distinguish between the diffuse and restricted components of EA. IgG antibody titers to EBNA were determined with the anticomplement technique (Reedman and Klein, 1973).

Statistical Analysis:

The chi-squared test and Student's *t* test were used to compare proportions and means, respectively.

Results

The mean age of the patients was 27.9 (±7.8 years) and of the controls, 30.7 (±3.5). There were 5 males and 27 females in each group.

Twenty-six patients had bilateral headaches and 6 had unilateral headaches. The headaches were temporal (17), frontal (6), occipital (2), parietal (1), or generalized (6). Twenty-eight patients described their headaches as steady, 2 as pounding, and 2 could not describe the quality of the headaches. Seventeen patients were evaluated for time of onset: the headaches started in the morning in 13 patients and in the afternoon or evening in 4. Fifteen patients described their headaches as severe and 17 as moderately severe. Nineteen (59%) of the 32 patients had associated neurological symptoms. Thirteen described paresthesias, 10 had tinnitus and/or dizziness, and 4 reported visual disturbances. Twenty-five patients complained of severe fatigue, and 11 had symptoms of depression. Twelve patients, but none of the controls, had a history of acute infectious mononucleosis. Three patients, but none of the controls, had a positive Monospot test. The results of EBV serology, dot hybridization, and clinical data on 32 patients with NDPH are summarized in Table III-1.

Two patients and one control had detectable titers of IgM to VCA. The DNA-DNA dot hybridization for EBV was positive in DNA extracted from oropharyngeal cells in 20 (60%) of the headache patients and 4 (12%) of the control subjects ($p < 0.001$; Figure III-1). The mean (± SE) titer for IgG against VCA was significantly higher in patients (537 ±75) than in controls (206 ±43) ($p < 0.01$). The IgG against VCA was >1:1000 in 13 headache patients and in only 2 of the controls. Similarly, the mean time (± SE) for EA was significantly higher (42.9 ±10.2) in patients than in controls (5.0 ±1.8) ($p < 0.001$). The IgG against EA was >1:32 in 18 patients and 2 controls (Figure III-2). Six VCA-seropositive patients had no detectable anticomplement IgG antibodies to EBNA. They did not have positive Monospot tests, atypical lymphocytosis, or clinical evidence of acute infectious mononucleosis. Only 1 VCA-seropositive control had no detectable anticomplement IgG antibodies to EBNA.

Seventeen patients were followed up for 3 to 18 months after the initial evaluation. Twelve of these initially had dot-blots positive for EBV, and 6 became negative during the follow-up period. Five of the 6 improved, but 1 patient continued to have headaches. The 6 who remained symptomatic continued to shed EBV in the oropharyngeal cells. Five of these 6 patients continued to have headaches, and 1 improved. Of the 5 patients who initially had negative dot-blots, 3 improved and 2 continued to have headaches, even though the dot-blots for EBV remained negative during the follow-up period.

Discussion

The syndrome of headaches, fatigue, and depression is commonly seen in clinical practice and is often attibuted to neurosis. In some cases these symptoms are associated with serological evidence of EBV reactivation (Tobi *et al.*, 1982; Jones *et al.*, 1985; Straus *et al.*, 1985). EBV is known to be associated with neurological signs and symptoms (Silverstein *et al.*, 1972; Grose *et al.*, 1975), and it has also been implicated as an etiological factor in affective disorders (Cadie *et al.*, 1976; Gotlieb-Stematsky *et al.*, 1981; Allen and Tilkian, 1986).

To our knowledge, the study reported here is the first to document an association with EBV in a subgroup of patients with chronic idiopathic headaches. Twenty-seven (84%) of the 32 patients with new daily persistent headaches and 8 (25%) of 32 controls had evidence of active EBV infection as demonstrated serologically and/or by dot hybridization of EBV DNA in oropharyngeal cells from throat washings. Six patients had no detectable IgG to EBNA, possibly an indication of an EBV-antigen-specific immunodeficiency leading to chronicity of EBV infections (Miller *et al.*, 1985). Recent reports suggest a possible role for herpes simplex virus in the pathogenesis of some cases of cluster headaches (Joseph and Rose, 1985; Hardebo, 1986). It is conceivable that EBV has a similar role in the pathogenesis of NDPH. In two studies, 35-73% of patients with serological evidence of EBV reactivation were found to have headaches and/or paresthesias (Jones *et al.*, 1985; Straus *et al.*, 1985). These reports and our findings that most patients with NDPH had evidence of active EBV infection suggest that headaches may be a relatively common clinical feature of EBV reactivation. Stress is known to precipitate transient immunosuppression (Bartrop *et al.*, 1977; Jemmott *et al.*,

1983), and EBV can be reactivated during immunosuppression (Strauch *et al.*, 1974; Glaser *et al.*, 1985). It is possible that the increased frequency of EBV reactivation in patients with NDPH is secondary to the stress resulting from chronic headaches. If this is so, EBV reactivation has no pathogenic role in NDPH. EBV reactivation is seen in a high proportion of patients with NDPH, but the precise role of the virus in headache syndromes remains to be elucidated.

Table III-1

Results of EBV Serology, Dot Hybridization, and Clinical Data on 32 Patients with NDPH

Age/sex	Past History of IM	Duration of headaches (mo)	VCA IgM	VCA IgG*	EA**	EBNA+	Monospot	EBV dot hybridization in oropharyngeal cells	Other symptoms
18/M	No	7	–	1:80	1:32	≥1:2	–	–	Fatigue
28/F	No	6	+	1:160	<1:8	1:8	–	+	Fatigue
25/M	12 mo	4	–	≥1:1000	<1:8	<1:2	–	+	Fatigue
39/F	No	2	–	1:160	<1:8	≥1:2	–	–	Nausea
15/F	8 mo	8	–	≥1:1000	1:32	1:8	–	+	Fatigue
30/F	12 mo	12	–	1:160	1:256	≥1:32	+	+	Depression, fatigue paresthesias
30/F	7 yr	3	–	≥1:1000	>1:8	<1:2	–	+	Depression, fatigue
52/M	10 yr	12	–	≥1:1000	1:32	<1:2	–	+	Depression, fatigue
23/F	No	2	–	1:160	1:128	1:8	–	+	Fatigue
30/F	No	1	–	1:160	1:32	1:8	–	–	Depression, dizziness fatigue
20/F	No	18	+	1:20	1:128	≥1:2	–	+	Paresthesias, fatigue dizziness, deficient concentration
37/F	No	5	–	1:160	<1:8	≥1:32	–	+	Paresthesias, fatigue, deficient concentration, depression
21/M	No	9	–	1:20	<1:8	<1:2	–	+	Double vision
27/M	No	8	–	≥1:1000	1:8	<1:8	–	–	Tremors, fatigue paresthesias
15/F	No	6	–	<1:20	<1:8	<1:2	–	–	Paresthesias, fatigue
38/F	No	4	–	≥1:1000	1:32	1:8	–	+	Fatigue
30/F	No	6	–	≥1:1000	<1:8	≥1:32	–	+	Dizziness, tinnitus, fatigue
20/F	No	4	–	≥1:1000	1:32	≥1:32	–	+	Paresthesias, blurred vision, fatigue, depression
24/F	2 yr	24	–	1:160	<1:8	≥1:32	+	–	Paresthesias, fatigue, dizziness
29/F	No	7	–	≥1:1000	<1:8	1:32	–	+	Lymphadenopathy, depression
25/F	No	14	–	1:160	1:32	≥1:32	–	+	Depression
28/F	10 yr	18	–	1:640	1:128	≥1:32	+	–	Dizziness, fatigue
33/F	3 yr	12	–	1:640	1:128	≥1:32	–	–	Paresthesias, fatigue
19/F	No	12	–	≥1:1000	<1:8	1:8	–	+	Blurred vision, fatigue
35/F	1 yr	12	–	≥1:1000	1:32	≥1:32	–	–	Paresthesias, fatigue
20/F	3 yr	15	–	1:160	1:32	<1:2	–	+	Fatigue, depression
40/F	No	6	–	≥1:1000	1:32	1:8	–	+	Paresthesias, fatigue, dizziness
32/F	2 yr	24	–	≥1:1000	1:32	≥1:32	–	+	Depression, dizziness
26/F	3 yr	6	–	1:160	≥1:8	1:32	–	+	Depression, fatigue
24/F	No	10	–	1:160	1:32	<1:2	–	–	Hemianopsia, paresthesias fatigue, dizziness
32/F	No	4	–	1:20	1:128	≥1:32	–	–	Paresthesias, fatigue dizziness

IM = infectious mononucleosis.
* IgG titers to VCA were calculated by testing serum dilutions of 1:20, 1:80, 1:160, 1:640, and 1:1000.
** Serum dilution started at 1:8.
\+ IgG titers to EBNA determined at dilutions of 1:2, 1:8, and 1:32.

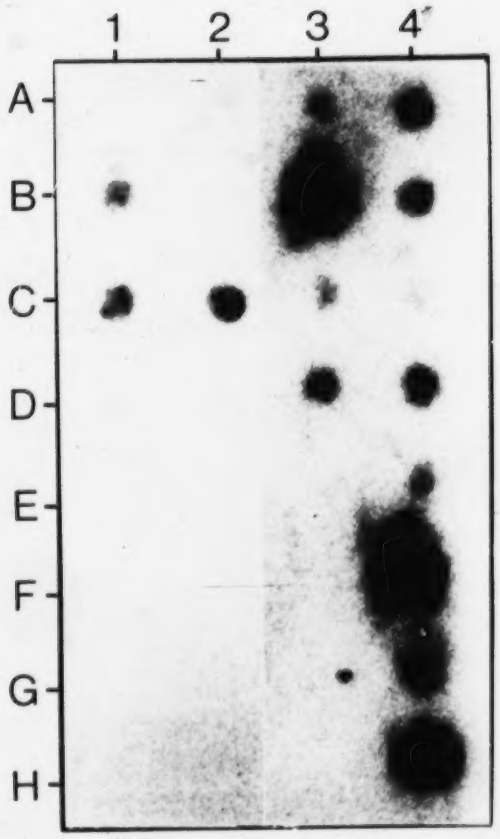

Figure III-1. Autoradiograph of DNA from oropharyngeal cells of patients and controls. Controls: *A1, A2, B2, E3,* and *F3* are dot negative; *B*1 and *E*4 are EBV positive. Patients: *D2* is EBV negative; *A3, A4, B3, B4, C1, C2, C3, C4, D1, D3, D4, F4, G4,* and *H4* are EBV positive. 5 µg of DNA extracted from fibroblasts infected with cytomegalovirus (*E1, E2*) and 5 µg of human placental DNA (*F1, F2, G1, G2*) were used as negative controls.

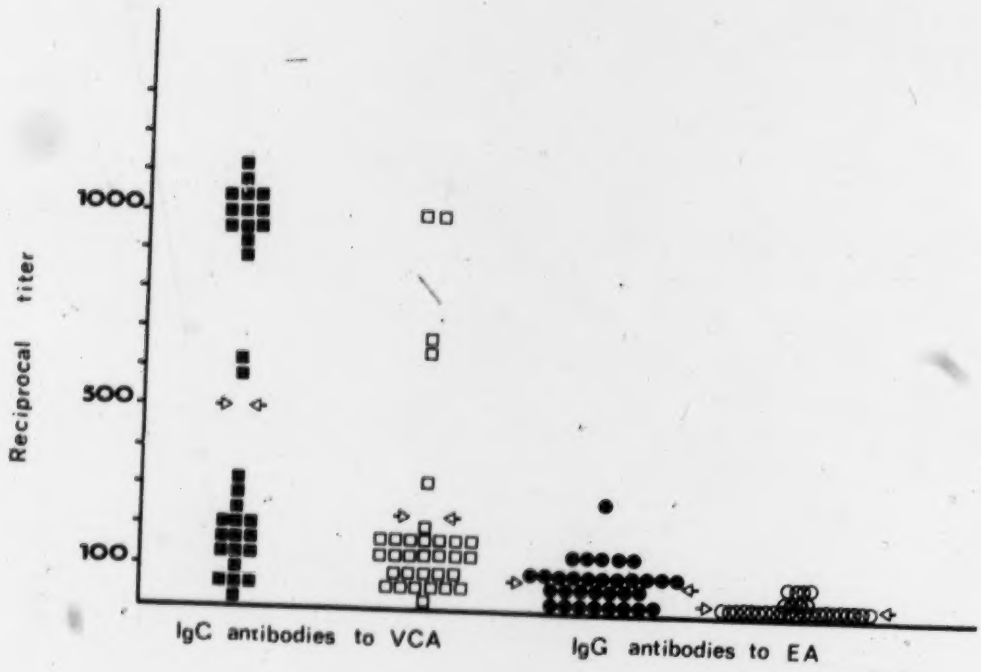

Figure III-2. Reciprocal IgG titers to EBV antigens from patients (closed squares, closed circles) and controls (open squares, open circles). Arrows represent geometric means. Analysis of variance was significant with an F-probability of <0.0005. Significance of difference between patients and controls: $p < 0.001$ for antibodies to EA, and $p < 0.01$ for antibodies to VCA.

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CHAPTER IV

HIGH LEVELS OF EPSTEIN-BARR VIRUS IN THE OROPHARYNX: A PREDICTOR OF DISEASE PROGRESSION IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

A VERSION OF THIS CHAPTER IS IN PRESS as, "High levels of Epstein-Barr virus in the oropharynx: A predictor of disease progression in human immunodeficiency virus infection." F. Diaz-Mitoma, A. Ruiz, G. Flowerdew, S. Houston, B. Romanowski, T. Kovithavongs, J. Preiksaitis, D.L.J. Tyrrell. *J. Med. Virol.*, 1990.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous virus that infects oropharyngeal epithelial cells (Sixbey *et al.*, 1984) and epithelial cells of the cervix (Sixbey *et al.*, 1986), and also immortalizes B cells (Chang and Golden, 1971). The normal host immune response will control EBV replication; however, the virus remains within some host cells in a latent form. Intermittent viral reactivation can occur, but it is generally subclinical. Reactivation has been observed more frequently in the immunocompromised host (Strauch *et al.*, 1974; Chatterjee and Chang 1982). EBV has been associated with Burkitt's lymphoma, nasopharyngeal carcinoma, and lymphomas in immunocompromised individuals (Miller, 1980). It has recently been associated with Hodgkin's lymphoma (Mueller *et al.*, 1989), non-Hodgkin's lymphoma in homosexual men (Purtilo *et al.*, 1985; Groopman *et al.*, 1986), and with interstitial pneumonia in children with AIDS (Andiman *et al.*, 1986). Uncontrolled EBV infection in severe immunodeficiency states may be associated with fever, leukopenia, intensified immunosuppression, widespread lymphoproliferation, and the development of lymphomas (Cleary *et al.*, 1986; Ho *et al.*, 1985).

It is estimated that at least 10% of HIV-seropositive asymptomatic individuals and up to 25% of symptomatic individuals will develop AIDS within three years (Fauci and Lane, 1987). The factors identified as being associated with progression of HIV infection include a decreased number of T-helper lymphocytes, an increased number of T suppressor cells, low titers of HIV antibody, high titers of CMV

antibody, sex with someone with AIDS or who has later developed AIDS (Polk *et al.*, 1987), an increase in levels of HIV antigens (Lange *et al.*, 1986), and oral hairy leukoplakia (Greenspan *et al.*, 1987). The interaction of viruses may play a role in the pathogenesis of AIDS (Armstrong, 1984; Hirsch, 1984). Homosexual men are particularly prone to acquiring infections by viruses of the Herpesviridae family, HIV, and hepatitis B virus. In this study we report on the prevalence and levels of EBV excretion in homosexual and heterosexual men. Our studies indicate that high levels of EBV excretion may be a good predictor of progression of HIV-induced disease in a subgroup of HIV-seropositive individuals.

Materials and Methods

Population:

The population for this prospective study consisted of 52 homosexual men attending a sexually transmitted disease (STD) clinic. Patients were classified according to the CDC classification system for HIV infections (Morbidity and Mortality Weekly Report, 1986). They included healthy men who were seronegative for HIV (Group I, $n = 25$), asymptomatic men who were seropositive for HIV (Group II, $n = 16$) and HIV-seropositive men with generalized lymphadenopathy (Group III, $n = 11$). Patients with Group IV (AIDS) disease were excluded from the study. We also studied 52 age-matched (±5 years) heterosexual male controls recruited from the same STD clinic. Complete histories and physical examinations were done initially and at 2, 6, 12, and 18 months. Progression of HIV infection was defined as the clinical deterioration of

HIV-seropositive individuals which made necessary a change of HIV disease group according to the CDC criteria for classification of HIV infection.

The initial laboratory assessment in all patients included a complete blood count and differential, a chemical profile, hepatitis B serology, and HIV serology performed by ELISA (DuPont de Nemours & Co., Wilmington, Del.) and confirmed by Western Blot (Bio-Rad, Hercules, Calif.). HIV p24 core antigen was determined by ELISA. Throat washings for EBV detection were performed initially and repeated at the two-month and one-year follow-up. IgG and IgM antibodies for CMV were done on the initial visit.

T4/T8 Ratios:

Homosexual men had T4/T8 ratios determined on the initial visit. This was determined by a complement-dependent ^{51}Cr release technique. Peripheral blood lymphocyte targets were lysed with the monoclonal antibodies 66.1 (against T4) and 51.1 (against T8) in the presence of Lowtox-H rabbit complement (Cedarlane, Hornby, Ontario). The percentages of specific ^{51}Cr release were used to calculate T cell subset ratio (Kovithavongs *et al.*, 1987).

Epstein-Barr Virus Detection:

A DNA-DNA dot hybridization assay was used to detect the levels of EBV excretion in throat washings. The dot hybridization for EBV has a sensitivity and specificity comparable to the lymphocyte transformation assay (Diaz-Mitoma *et al.*, 1987). In this assay we used a DNA probe consisting of the *Bam*HI-W (3.0 kb) fragment from the internal repeat 1 (IR1) and the EcoR1-*Bam*HI (4.0 kb) subgenomic fragment adjacent to the left telomeric sequence of EBV. Levels of EBV DNA were determined by measuring the density of autoradiographs of the

filters probed with the EcoR1-*Bam*HI 4.0 kb subgenomic fragment. The *Bam*HI-W probe can be used to detect EBV excretion, but is not used in quantitative studies since the copy number of this repeating sequence varies in different strains (Bornkamm *et al.*, 1989). We therefore used the EcoRI-*Bam*HI 4.0 kb fragment for the quantitative assay.

After swabbing the oropharynx, the patients gargled with 20 ml of saline for approximately 20-30 seconds. Cells were pelleted from the saline solution. Cellular DNA was extracted and spotted onto nylon filters. The probes were radiolabeled with (^{32}P)-dCTP by nick translation and were hybridized to the filters as previously described (Diaz-Mitoma *et al.*, 1987). The viral DNA was detected by autoradiography of the filters and quantitated by densitometry scanning. An estimate of EBV DNA from cellular extracts was obtained by comparison to serial dilutions of purified B95-8 EBV DNA (0.05, 0.25, 0.5, 1, and 10 ng). Positive controls for dot hybridization consisted of serial dilutions of DNA from B95-8 and Raji cells. Negative controls were DNA from cord lymphocytes and DNA from human lung fibroblasts infected with CMV. Two standard deviations above the mean for negative controls was used to define the cutoff point between positive and negative dot hybridization. The median of the levels of EBV DNA in HIV-seropositive homosexual men was used to define the cutoff point between "high EBV excreters" and "low EBV excreters." The amount of human DNA extracted from throat washings was measured by dot hybridization and densitometry scanning. The probe to detect cellular DNA was human placental DNA radiolabeled with (^{32}P)-dCTP by nick translation to a specific activity of 10^7 cpm/μg of DNA. Hybridization conditions in this experiment were the same as for the detection of viral DNA.

EBV Serology:

EBV IgG antibodies to the viral capsid antigen (VCA), early antigens (EA), and nuclear antigens (EBNA) were measured in patients and controls by an enzyme-linked immunosorbent assay (Dupont Co., Billerica, Mass.) (Luka *et al.*, 1984).

CMV Serology:

CMV antigen for IgG and IgM assays was prepared by sonication of CMV AD169-infected MRC-5 cells. CMV-specific IgG was determined by ELISA (Preiksaitis *et al.*, 1988). All specimens were screened at a 1:50 dilution. Quantitative titers were determined by comparing optical density (OD) readings obtained at 1:1,000 and 1:100,000 serum dilutions to a standard curve. CMV-specific IgM was measured by ELISA (Behring Diagnostics). An optical density of ≥200 was considered positive.

Statistical Analysis:

Many of the variables had highly skewed distributions which precluded the use of *t* tests and other parametric procedures. For example, the number of sex partners was highly skewed to the right. For presentation and analysis, the data were categorized as shown in Table IV-1. Comparisons between different groups of subjects were carried out using Fisher's exact test or the Mantel-Haenzel chi-squared test for trend (Mantel, 1963). All *p* values quoted are for a two-sided test of significance.

Proportional hazards regression analysis (Cox, 1972) was used to determine the best predictors of progression of HIV infection. Although most of the subjects in the study were followed for 18 months, a few were followed for

slightly longer while others were followed for a shorter period. The proportional hazards procedure has the flexibility of allowing for variable lengths of follow-up and also allows for several predictors to be examined simultaneously so that their independent effects can be evaluated. The method is often described as quasi-parametric since it is based on the ranks of the times to progression. The predictors do not have to be categorical. In our analysis a square root transformation was applied to the EBV DNA and HIV p24 core antigen values so that their distributions would be more symmetrical. (The statistical analysis of this project was performed by Dr. Gordon Flowerdew from the Department of Health Sciences Administration and Community Medicine, University of Alberta.)

Results

The breakdown of subjects by age, number of sex partners in a lifetime, occurrence of STDs, and by various markers of immunosuppression is shown in Table IV-1. The ages of the heterosexual and homosexual men were similar (30 ±8 vs. 31 ±9). The median number of sex partners in a lifetime was 50 (range: 4-2,500) for the homosexual men and 12 (range: 2-300) for the heterosexual men ($p < 0.0001$). There was a trend towards more sex partners in HIV-seropositive than in HIV-seronegative homosexual men [median: 100 (range: 6-2,500) vs median: 30 (range: 4-500), respectively]. The number and frequency of sexually transmitted diseases was higher in homosexual than in heterosexual men. A history of an infectious mononucleosis-like syndrome and gonorrhea were more prevalent in the homosexual population. Fourteen of the 52 homosexual men and 4 of the 52 heterosexual men had a prior history of an infectious

mononucleosis-like syndrome ($p = 0.018$). There were 28 homosexual men and 10 heterosexual men who had had one or more episodes of gonorrhea ($p = 0.0005$). Seven homosexual and two heterosexual men had a history of syphilis. There were 19 homosexual and 19 heterosexual men with one or more episodes of non-gonococcal urethritis. None of the patients in this study had hairy leukoplakia.

Sample autoradiograms of the filters containing DNA from throat washings from heterosexual and homosexual men are shown in Figure IV-1. Densitometry scanning of the "dots" representing EBV and human DNA (hDNA) allowed us to estimate the amount of EBV DNA in nanograms per microgram (npm) of cellular DNA (Figure IV-2).

The presence of EBV DNA in throat washings was more common in homosexual (38 of 52) than in heterosexual men (20 of 52) ($p < 0.001$). Among homosexuals, the presence of EBV DNA was more common in HIV-seropositive (24 of 27) than in HIV-seronegative homosexual men (14 of 25) ($p = 0.018$). Similar results were found with regard to the amount of EBV excreted. Homosexuals had a higher median EBV excretion than heterosexuals [median: 0.53 npm (range: 0.02-5.8) vs median: 0.27 npm (range: 0.03-1.73), $p = 0.0001$]. Among homosexuals, HIV-seropositive men had a higher median EBV excretion than HIV-seronegative men [median: 0.63 npm (range: 0.03-5.8) vs median: 0.26 npm (range: 0.02-4.5), $p = 0.008$]. Among HIV-seropositive men, those in Group III had a higher median EBV excretion than those in Group II [median: 0.81 npm (range: 0.03-5.8) vs median: 0.53 (range: 0.04-1.18)]. EBV excretion is clearly a sensitive marker of underlying immunosuppression. Other markers such as HIV p24 core antigen,

T4/T8 ratio, IgG and IgM to CMV, and IgG to EBNA were also examined and their results are shown in Table IV-1.

We examined the ability of the different markers to predict the clinical progression of the HIV infection. The number of subjects progressing within a 12- to 24-month period out of the 27 HIV-seropositive homosexual men was noted and tabulated by various categories of the predictors (Table IV-2). The median time of follow-up in HIV-seropositive individuals in whom progression was demonstrated was 15 months (range: 12-20 months), and the median time of follow-up in HIV-seropositive individuals in whom progression was not demonstrated was 14 months (range: 12-24 months). Nine of the 27 HIV-seropositive homosexuals progressed: 3 from Group II to Group III, 1 from Group II to Group IV, and 5 from Group III to Group IV. All 9 subjects had high levels of EBV excretion (> 0.63 npm). In contrast, only 3 of the 18 subjects who did not progress had high levels of EBV excretion ($p < 0.001$) (Figure IV-2). Seven of the 9 subjects who progressed had a detectable level of HIV p24 core antigen. In contrast, only 2 of the 18 subjects who did not progress had detectable HIV p24 core antigenemia ($p < 0.003$). Similar, though less striking, results were noted for the T4/T8 ratio, IgG to CMV and IgM to CMV. In the case of the T4/T8 ratio, the association was non-significant, perhaps due to the fact that not all subjects had the T4/T8 ratio measured on the first visit.

We also examined the predictors as continuous variables using proportional hazards regression, thereby eliminating the use of arbitrary cutoff points between high and low levels of each predictor. EBV excretion was found to be the best single predictor of progression of HIV infection ($p < 0.001$). HIV p24 core

antigenemia (p = 0.048) and low EBNA (p = 0.024) were also significant predictors and independent of EBV excretion, although low EBNA by itself was not a significant predictor.

All homosexual men were EBV-seropositive. Among the heterosexual men there were 50 individuals who were EBV-seropositive and 2 individuals who had negative serology for VCA and EBNA-IgG. The levels of VCA-IgG were not statistically different in heterosexual and homosexual men [mean optical density (OD) = 1.30 ±0.58 vs. 1.17 ±0.37 (paired t test, $p > 0.05$)]. There was no difference in the levels of VCA-IgG in HIV-seropositive or HIV-seronegative homosexual men (mean OD = 1.19 ±0.39 vs 1.13 ±0.28, $p > 0.1$). Four of 52 homosexual men (3 HIV-seropositive and 1 HIV-seronegative) and 1 of 52 heterosexual men had positive ELISAs for EA-IgG. The prevalence of IgG to CMV was higher in homosexual (48 of 52) than in heterosexual men (29 of 52, x^2 = 18, p = 0.0001). There was no significant difference in the titers of IgG to CMV between HIV-seropositive (38,555 ±29,803) and HIV-seronegative homosexual men (32,100 ±24,812). Heterosexual men had lower IgG titers to CMV (9,462 ±15,774, p = < 0.0001). The titer of IgG to CMV in HIV-seropositive individuals who demonstrated progression was not different from the titer of IgG to CMV in individuals not demonstrating progression of HIV infection. Fifteen (28%) of 52 homosexually active and 5 (10%) heterosexually active men were positive for IgM to CMV (Fisher's exact test, p = 0.011). Eleven (40%) of the 27 HIV-seropositive and 4 (16%) of the 25 HIV-seronegative homosexual men had positive IgM to CMV (Fisher's exact test, p = 0.047). Five (55%) of the 9 individuals who

progressed to Group III or IV HIV infection and 6 (33%) of 18 patients with no progression of HIV infection demonstrated IgM to CMV (Fisher's exact test, $p = 0.24$).

Discussion

More than 95% of the human population worldwide is EBV-seropositive by the time they reach adulthood and 20-60% of healthy asymptomatic adults screened at any one time shed EBV at low levels (Yao *et al.*, 1985). There have been several studies suggesting that herpesviruses may be co-factors in the progression of HIV disease (Lange *et al.*, 1986; Montagnier *et al.*, 1984; Casareale *et al.*, 1984; Blumberg *et al.*, 1987; Gendelman *et al.*, 1986). Homosexual men are nearly 100% seropositive for EBV (Sumaya *et al.*, 1986; Rahman *et al.*, 1989; Nerurkar *et al.*, 1987). HIV-seropositive men have higher EBV antibody titers than HIV-seronegative individuals (Rahman *et al.*, 1989). In addition, two studies have shown that EBV excretion occurs in 58-76% of homosexual men (Sumaya *et al.*, 1986; Crawford *et al.*, 1984). Alsip and coworkers found higher levels of EBV excretion in patients with AIDS than in patients with ARC or acute infectious mononucleosis (Alsip *et al.*, 1988).

The detection of EBV-DNA by dot hybridization has a sensitivity of 90% and a specificity of 98% when compared to lymphocyte transformation, and in immunocompromised patients the detection of EBV by dot hybridization was a more reliable indicator of EBV reactivation than EBV-specific serology (Diaz-Mitoma *et al.*, 1987). Chang *et al.* (1985) also found that the reliability of the

interpretation of EBV serology in the diagnosis of EBV reactivation was low. Although slot-blots were specifically designed for quantitative assays, in our hands the dot-blot assay was an accurate and reproducible test. Using the dot hybridization, we not only detected reactivation of the virus but could also approximate the amount of virus excreted. The densitometry scans of the "dots" permitted us to categorize the reactivators into "high" and "low" excreters. This has proven useful since the "high" excreters of EBV form a subgroup of patients with a high probability of HIV progression. This is the first report that associates high levels of EBV excretion and rapid progression of HIV-induced disease. Although an increase in excretion of EBV is an expected finding in HIV-seropositive individuals who are immunosuppressed, high levels of EBV excretion were detected prior to significant differences in the T4/T8 ratios. In addition, high EBV excretion was also found in some heterosexual males and HIV-seronegative homosexual patients. This would suggest that EBV reactivation is not purely on the basis of HIV-induced immunosuppression. High and low EBV excreters have also been described in the general population (Yao *et al.*, 1985). It may be that individuals with high EBV excretion are predisposed to a more aggressive course once they acquire HIV.

Although an increased incidence of EBV-associated lymphomas is observed in HIV-seropositive individuals (Groopman *et al.*, 1986), none of our patients developed lymphomas during the follow-up period.

Several studies suggest that the number of T4 and T8 cells, HIV p24 core antigenemia, high IgG or IgM titers to CMV, and the presence of hairy leukoplakia are predictors of progression of HIV-induced disease (Polk *et al.*, 1987; Lange *et*

al., 1986; Greenspan *et al.*, 1987). In this study, high levels of EBV excretion and HIV p24 core antigenemia were the best predictors of progression of HIV infection. In contrast, CMV serology was not a good predictor of progression. Hairy leukoplakia may be a good predictor of progression when it exists; however, it was not present in any of our patients. We could not demonstrate a statistical difference in the T4/T8 ratios between individuals who had disease progression and those patients who were clinically stable during the study. Perhaps this was due to lack of testing in all subjects. However, decreasing T4/T8 ratios may be a late manifestation of HIV infection. HIV infection induces severe immunological abnormalities in T cells, B cells, and antigen-presenting cells early in infection before CD4+ T cell numbers start to decline (Miedema *et al.*, 1988).

The association between EBV infection and HIV expression has been previously studied. The first report of persistent HIV infection *in vitro* was in a lymphoblastoid cell line transformed by EBV (Montagnier *et al.*, 1984). Although HIV can infect both EBV-infected and -uninfected B cells, the production of HIV may be increased by EBV infection (Monroe *et al.*, 1988). HIV is cytopathic to T lymphocytes; however, EBV-infected lymphoblastoid cell lines may release retroviral particles without undergoing cytolysis (Casareale *et al.*, 1984; Monroe *et al.*, 1988). HIV infection of EBV-infected B lymphocytes may serve as a continuous source of HIV, which might amplify the cycle of HIV infection.

One mechanism by which EBV could increase HIV production is through promoter transactivation. Kenney and coworkers have found that an immediate-early gene of EBV can increase the level of gene expression of HIV (Kenney *et*

al., 1988). A gene product of herpes simplex I can increase the expression of a heterologous gene linked to the HIV promoter (Mosca *et al.*, 1987). Cytomegalovirus infection may increase the expression of HIV *in vitro* (Skolnik *et al.*, 1988). Other DNA viruses such as papovavirus may also transactivate the HIV promoter (Gendelman *et al.*, 1986). However, since EBV is a systemic virus infection that infects lymphocytes and epithelial cells, it may be of more significance in the pathogenesis of HIV disease.

In conclusion, our data suggest that high levels of EBV excretion are a marker or predictor of progression of disease in a subset of HIV-seropositive individuals. In this study, high levels of EBV excretion had similar predictive values to HIV p24 core antigenemia and better positive and negative predictive values than several other predictors of progression of HIV-induced disease. The actual role that EBV plays in the pathogenesis of HIV infection is not known. Nonetheless, our results suggest that EBV may be of significance in the natural history of HIV infection. Prophylactic chemotherapy directed against viral co-factors may be an important approach to the slowing of progression of disease in this high-risk group.

Table IV-1

Breakdown of Subjects by Age, Number of Sex Partners, STD History, and by Various Markers of Immunosuppression

		Homosexual Men					Heterosexual Men	
		HIV-	HIV+					
		n=25	(Group 2) n=16	(Group 3) n=11	p*	Total n=52	n=52	p**
Age	20-29:	13	7	7	NA	27	29	NA
	30-39:	6	5	3		14	13	
	40-50:	6	4	1		11	10	
Number of sex partners	0- 9:	3	3	0	NS	6	16	.0001
	10- 49:	11	1	6		18	26	
	50-199:	8	6	2		16	8	
	≥200:	3	6	3		12	2	
Hx infectious mononucleosis	yes:	4	4	6	NS	14	4	.018
	no:	21	12	5		38	48	
Hx gonorrhea	yes:	9	10	9	.027	28	10	.0005
	no:	16	6	2		24	42	
Hx syphilis	yes:	3	2	2	NS	7	2	NS
	no:	22	14	9		45	50	
Non-gonococcal urethritis	yes:	12	5	2	NS	19	19	NS
	no:	13	11	9		33	33	
EBV DNA+	.00:	11	2	1	.008	14	32	.0001
	.01-0.49:	9	7	4		20	16	
	.50-1.99:	4	7	3		14	4	
	≥2.00:	1	0	3		4	0	
HIV p24 core antigenemia	negative:	25	11	7	.003	43	ND	
	positive:	0	5	4		9	ND	
T4/T8 ++	.00-0.99:	4	5	6	.040	15	ND	
	≥1.00:	10	4	0		14	ND	
CMV IgG	negative:	1	2	1	NS	4	29	.0001
	positive:	24	14	10		48	23	
CMV IgM	negative:	21	11	5	NS	37	47	.024
	positive:	4	5	6		15	5	
EBNA IgG (OD)	.00-0.99:	10	4	3	NS	17	11	NS
	≥1.00:	15	12	8		35	4	

(*) *p* value comparing HIV-seronegative and HIV-seropositive men
(**) *p* value comparing Homosexual and Heterosexual men
(+) nanograms of EBV DNA per microgram of human DNA
(++) T4/T8 ratios were not measured in all patients
NA = non-assessable
NS = non-significant
ND = not done

Table IV-2

Evaluation of Markers of Immunosuppression as Predictors of Progression of HIV-Induced Disease in a Cohort of 27 HIV-Seropositive Homosexual Men

		Progression	Non-progression	
EBV DNA*	.00:	0	3	$p < .0003$
	.01-0.49:	0	11	
	.50-1.99:	6	4	
	≥ 2.00:	3	0	
HIV p24 core antigenemia	negative:	2	16	$p < .003$
	positive:	7	2	
T4/T8**	.00-0.99:	5	6	NS
	≥ 1.00:	3	1	
CMV IgG	negative:	1	2	NS
	positive:	8	16	
CMV IgM	negative:	4	12	NS
	positive:	5	6	
EBNA IgG (OD)	.00-0.99:	3	4	NS
	≥1.00:	6	14	

(*) nanograms of EBV DNA per microgram of human DNA
(**) T4/T8 was not measured in all patients
NS = non-significant

Note that the *p*-values are for two-sided tests.

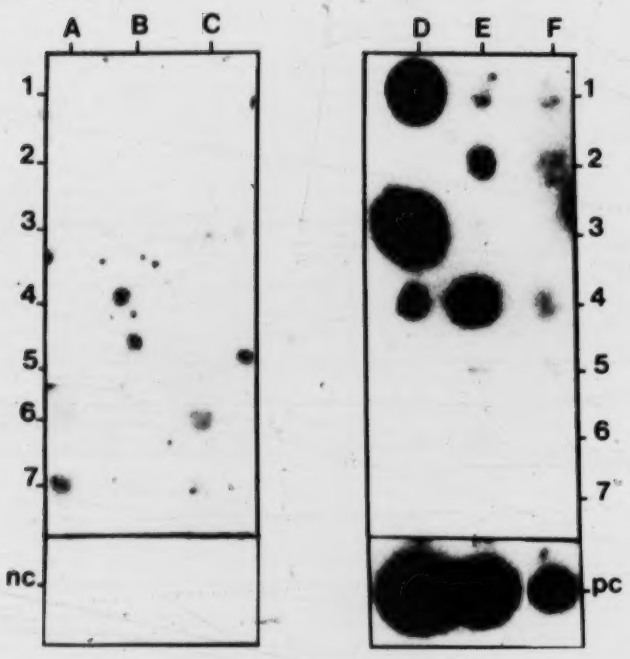

Figure IV-1. Nucleic acid dot hybridization with the purified ^{32}P-labeled *Bam*HI-W subgenomic fragment of EBV. DNA extracted from oropharyngeal cells of heterosexual (*A, B, C*), and homosexual (*D, E, F*) men. Dots *1B, 6A, 6C, 7A,7C* are positive for EBV DNA and belong to heterosexual men. Dots *1A, 1C, 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C, 5A, 5B, 5C, 6B, 7B* are negative for EBV DNA. Dots *1E, 1F, 2F, 4D, 5E, 6E* are positive for EBV and belong to HIV-seronegative homosexual men. Dots *1D, 2E, 3D, 4F, 4E* are positive for EBV DNA and are from HIV-seropositive homosexual men. Dots *5F, 6D, 7E* are negative for EBV DNA and are from HIV-seropositive homosexual men. Negative controls (nc): The dot *ncA* is the DNA extracted from one million cord blood lymphocytes. Placental human DNA (five micrograms) was spotted in *ncB* and *ncC*. Positive controls (pc): Titration of B95-8 EBV DNA. 10×10^{-9} g (*pcD*), 5×10^{-9} g (*pcE*), 1×10^{-9} g (*pcF*) of EBV DNA purified from virions of the producer cell line B95-8.

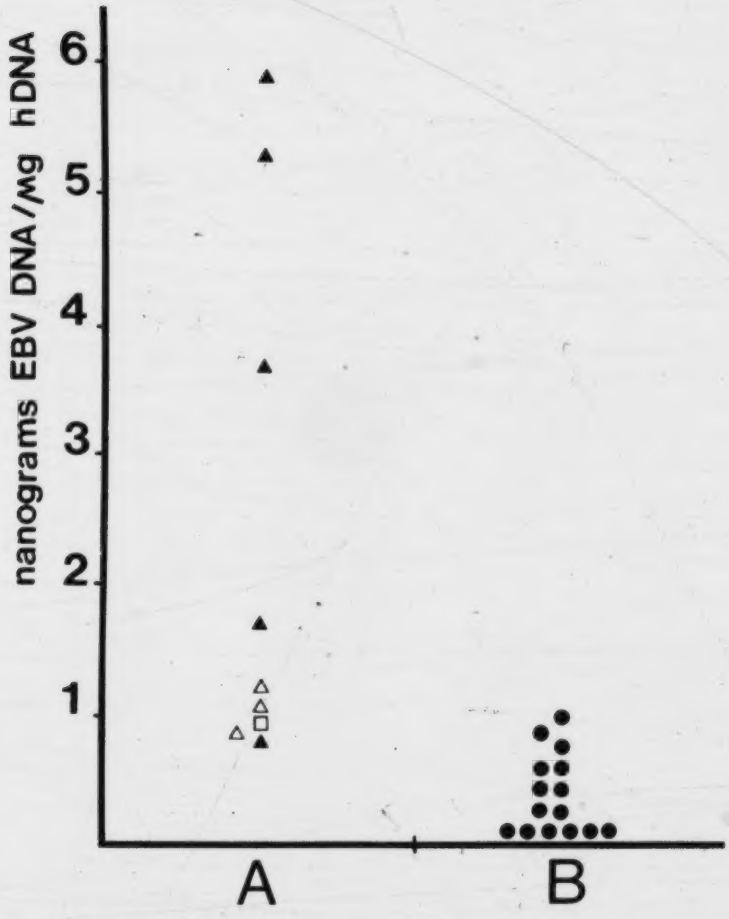

Figure IV-2. Levels of EBV DNA in throat washings from HIV-seropositive individuals with progression (*A*), and without progression (*B*) of HIV infection. Densitometry scanning from autoradiograms of the EBV dot hybridization assay used to quantitate EBV DNA in exfoliated oropharyngeal cells obtained from throat washings of 27 HIV-seropositive individuals. A 4.0 kb ^{32}P-labeled *Bam*HI-EcoR1 probe, which is a unique region adjacent to the left telomeric sequence of EBV, was used for the quantitation of EBV DNA. Twenty-four individuals were positive for EBV and 3 were negative for EBV. There were 9 individuals who demonstrated progression of HIV-induced disease during 18 months of follow-up (Group A). Five patients progressed from Group III (ARC) to Group IV (AIDS) (closed triangles), 3 patients progressed from Group II (asymptomatic) to Group III (open triangles), and 1 patient progressed from Group II to Group IV (open square). Eighteen patients remained clinically stable, 3 were EBV-negative and 15 were EBV-positive (Group B). Patients in Group A had higher levels of EBV DNA than patients in Group B (median: 1.58 vs 0.21 ng EBV DNA per µg of cellular DNA, respectively; ANOVA, $F=14.5$; $p < 0.001$).

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CHAPTER V

ANALYSIS OF THE TELOMERES OF EBV TO DEFINE THE INTRACELLULAR MOLECULAR CONFIGURATION OF THE VIRAL GENOME IN IMMUNOSUPPRESSED PATIENTS AND ITS IMPLICATION FOR ANTIVIRAL THERAPY

A VERSION OF THIS CHAPTER IS IN PREPARATION FOR PUBLICATION as, "Analysis of the telomeres of EBV to define the intracellular molecular configuration of the viral genome and its implication for antiviral therapy." F. Diaz-Mitoma, A. Ruiz, J.K. Preiksaitis, D.L.J. Tyrrell.

Introduction

After primary EBV infection, individuals remain life-long carriers. It is debatable whether EBV-seropositive individuals shed EBV persistently in the oropharynx (Yao *et al.*, 1985; Sixbey *et al.*, 1989), but at least 20% of such individuals shed detectable levels of EBV at any one time (Gerber *et al.*, 1972; Golden *et al.*, 1973; Diaz-Mitoma *et al.*, 1987). Where the virus replicates and in what form it persists remain incompletely answered questions.

EBV DNA is found primarily in desquamated oropharyngeal epithelium (Sixbey *et al.*, 1986), but it may also be present in other epithelial tissues such as the cervix (Sixbey *et al.*, 1986) and urethra (Sixbey *et al.*, 1989a). The virus genome has been found in the epithelium of the tonsils (Sixbey *et al.*, 1987) and the ductal epithelium of parotid gland biopsies by *in situ* hybridization (Wolf *et al.*, 1984). These studies suggest that the epithelium represents the primary site for permissive virus replication. During primary infection or reactivation, epithelial cells are the likely source of virions that may contribute significantly to viremia and the spread of the virus, causing infection of B cells and other tissues such as the lung, spleen, and liver (Andiman *et al.*, 1985; author's unpublished observations). During immunosuppression, the spread of EBV from permissively infected tissues may result in episodes of fever, further immunosuppression, and the induction of EBV-associated lymphomas (Cleary *et al.*, 1986). In HIV-seropositive individuals, EBV causes hairy leukoplakia (Greenspan *et al.*, 1985.

High levels of EBV replication in the oropharynx are associated with progressive HIV infection (Diaz-Mitoma *et al.*, 1990). Yao *et al.* (1985) have reported a direct correlation between the amount of virus excreted in the oropharynx and the number of EBV-positive peripheral lymphocytes. The determination of the level of virus present in the oropharynx, which we have referred to as the "virus load," may be important in assessing the risk of developing EBV-associated disease, and it may also be important as a predictor in the progression of HIV infection.

EBV has two distinct life cycles, a replicative and a latent life cycle. EBV causes latent infection of B lymphocytes. During latency, the EBV remains intracellular in an episomal configuration (Tanaka and Nonoyama, 1974). In contrast, linear forms of the viral genome, which are 172 kilobase double-stranded DNA molecules, are found during permissive infection. Linear forms of the genome are encapsidated into virions (Kinter and Sugden, 1979). These virions may produce a viremia and infection in other tissues during immunosuppression. The linear forms of the virus are susceptible to acycloguanosine, while the episomal forms found during latency are not susceptible to antiviral agents (Colby *et al.*, 1980; Katz *et al.*, 1989). After infection of immature oropharyngeal cells or B cells, linear forms circularize by the joining of the tandem repeat sequences at the ends of the genome. This process creates a fused terminal restriction fragment, which may also be detected in concatemeric molecules. On the other hand, linear molecules of EBV DNA will have smaller telomeric restriction fragments than the fragments of circular EBV DNA. In addition, the telomeric restriction fragments of linear DNA are heterogeneous as the number of 500 bp terminal repeats varies in each linear DNA molecule; therefore, a ladder of small restriction fragments is seen in Southern blot analysis of EBV DNA (Raab-Traub

and Flynn, 1986; Katz *et al.*, 1989). Figure V-I depicts the expected Southern blot bands from linear and episomal forms of the EBV genome. We studied the DNA extracted from oropharyngeal cells from HIV-seropositive individuals and from organ transplant recipients. We also analyzed the replicative forms of EBV in oropharyngeal cells in order to define whether there was a preponderance of forms containing joined termini (circular or concatemeric) or linear forms. If there is a preponderance of linear forms, this would suggest that there was permissive replication, and antiviral therapy may be of benefit in these patients.

Materials and Methods

Patients:

Seventeen patients who underwent cardiac transplantation were prospectively studied. We also studied one patient who developed a lymphoproliferative disorder after organ transplantation. Exfoliated oropharyngeal cells from 23 HIV-seropositive and 8 non-immunosuppressed individuals were analyzed. Oropharyngeal cells and serum samples were collected every 2-3 weeks for 3 to 6 months. During each of these visits, patients underwent clinical examinations and were evaluated for any symptoms or signs, particularly signs of rejection of the allograft, fever, or lymphadenopathy. Laboratory investigations included CBC with differential counts and hepatic and renal function tests.

Collection of Oropharyngeal Cells and Extraction of DNA:

Patients had thorough swabbing of the mouth surfaces, including the pharyngeal pillars, lateral aspects of the tongue, and the buccal mucosa.

Individuals were then asked to swish thoroughly and gargle with 20 ml of saline solution for approximately 30 seconds. The mouth washings were collected in 50-ml capped centrifuge tubes and centrifuged at 800 *g* for 10 mins. The pellets were collected and washed once in phosphate-buffered saline solution (PBS). This process yields a median of 1.6×10^6 cells (range: 1.6×10^5 to 7.3×10^6 cells). Total DNA was extracted from the cell pellets as previously described (Maniatis *et al.*, 1982). Briefly, the cell pellets were resuspended in proteinase K buffer (10 m*M tris*, pH 7.4, 10 m*M* EDTA, 150 m*M* NaCl) to which were added SDS (0.4 %) and proteinase K (1 mg/ml; Boehringer Mannheim, Dorval, Que.). The cells were incubated at 65°C for 15 mins and overnight at 37°C under gentle agitation. The DNA was treated with RNase A (DNase-free) and extracted 5 times with phenol:choloroform and was precipitated in 0.3 *M* Na acetate in cold ethanol. Dot hybridization was used to confirm the presence of EBV DNA. Total DNA was suspended in 25-50 µl of TE buffer and an aliquot spotted onto a nylon filter (Amersham, Oakville, Ont.) with a manifold apparatus (Bio-Rad, Mississauga, Ont.). The concentration of cellular DNA was measured by fluorimetry (Morgan *et al.*, 1979). One to 3 micrograms of oropharyngeal cellular DNA (after pooling several samples of cells from the same patient) were treated with *Bam*HI (Pharmacia, Uppsala, Sweden) using an enzyme concentration 4-6 times that recommended by the supplier and incubated at 37°C for 4-6 hrs. Total digestion of cellular DNA by the restriction enzyme was monitored by electrophoresis in a minigel. Agarose (Bio-Rad) gels (0.8%) were cast in a Tyler gel apparatus (Edmonton, Alta.) and run for 18-24 hrs at 40 v/h. The digested DNA was transferred to nylon filters by the procedure described in Southern *et al.* (1975), and was hybridized as described below.

Cell Lines:

Known EBV producers and latently infected cell lines were analyzed for circular and linear forms. The Raji cell line, which was originally isolated from a Burkitt's lymphoma, is latently infected with EBV and contains only episomal forms of the EBV genome (Tanaka and Nonoyama, 1974; Raab-Traub and Flynn, 1986). The P3HR-1 cell line is a subclone of a cell line (Jijoye) which was originally isolated from a lymphoma tissue, but, in contrast to the Raji cells, it releases EB virions. The B95-8 cell line consists of marmoset leukocytes infected with EBV. This cell line is permissive for virus replication and releases large amounts of virions.

The cell lines Raji and P3HR-1 were treated with inducers of EBV replication. Cells were exposed to *n*-butyrate (4 m*M*) and *n*-butyrate plus the tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA) (40 ng/ml) for 24 hrs before harvesting. The cells were grown in RPMI 1640 containing heat-inactivated fetal calf serum (10%), as previously described (Qualtière and Pearson, 1980). The cells were pelleted, and the DNA was extracted using the same techniques as described for oropharyngeal cells.

Purified EBV DNA was purchased from the Showa University Research Institute (St. Petersburg, Florida) and used as a positive control.

Probes and DNA Hybridization Studies:

We had previously found that some immunosuppressed patients excrete high levels of EBV in throat washings (Diaz-Mitoma *et al.*, 1987; 1990, see Chapters II and IV). In order to identify organ transplant recipients with levels of EBV excretion which were high enough to perform the Southern blots, throat washings were screened by dot hybridization. Filters were probed with the

*Bam*HI-EcoRi 4.0 kb fragment (non-repeating sequence), which is adjacent to the left EBV telomere, to estimate the quantity of EBV DNA as previously described (Diaz-Mitoma *et al.*, 1990, see Chapter VI). The amount of human DNA spotted in each dot was determined by probing the filters with an actin gene, followed by densitometry analysis of the dots. The actin gene is a 2.4 kb fragment, which was purified from the recombinant plasmid (kindly donated by Christine Boumah). The DNA probes used to detect the configuration of the EBV ends were isolated from the recombinant plasmid pSal-*Bam* ends, which was a generous gift from Dr. Bill Sugden (University of Wisconsin, Madison, Wis.) (see Figure V-2) (Sugden*et al.*, 1984). The *Bam*HI-H and W fragments were isolated from recombinant plasmids also provided by Dr. Sugden. DNA of the plasmid pSal-*Bam* ends was sequentially digested with Bgl II, EcoRI, and *Bam*HI (Figure V-2B). Isolation of the recombinant EBV subgenomic fragments was performed by cutting and extracting the DNA bands from low melting point agarose (Maniatis *et al.*, 1982). The purified DNA probes were radiolabeled with (^{32}P)-dCTP using the method of random oligopriming (oligopriming kit, Amersham, Canada). In a typical labeling reaction, 50 nanograms of DNA could be labeled to a specific activity of $>10^9$ cpm/µg of DNA. Prehybridization was performed for 6-8 hrs at 42°C in 50% formamide, 6 x SSC (1 x SSC = 0.15 *M* NaCl and 0.15 *M* sodium citrate), Denhardt's solution, 10% dextran sulfate, and 150 µg/ml of sheared and denatured salmon sperm DNA. 10^6 cpm/ml of the denatured probe was added to the hybridization solution and was incubated at 42°C overnight. Washings were performed at room temperature three times in 2 x SSC and 0.1% SDS and once in 0.1 x SSC at 62°C for 45 mins. Initially, filters were exposed to an X-OMAT AR5 film (Kodak, Rochester, N.Y.) overnight with intensifying screens. Longer exposures were usually necessary to visualize the bands by autoradiography

(24-72 hrs). Southern blots were probed with the *Bam*HI-W or *Bam*HI-H fragments to demonstrate the presence of EBV DNA in the filters. The blots were also hybridized with the 2.4 kb Bgl II-*Bam*HI EBV subgenomic fragment, which is a unique sequence adjacent to the right end of the EBV genome (Figure V-2). This fragment hybridizes to the right-terminal *Bam*HI fragment of EBV that contains approximately 3.5 kb of unique DNA and varying numbers of terminal repeats (TR). Some filters were hybridized with a 4.0 kb probe which represents the *Bam*HI-EcoRI fragment that is adjacent to the left end of the viral genome (Figure V-2). This probe identifies the left *Bam*HI terminus of EBV and contains approximately 4 kb of unique DNA and multiple copies of TR (Dambaugh *et al.*, 1980). The fused termini, containing 7.5 kb of unique DNA, could be identified in the Southern blots by their larger size.

Results

The Configuration of EBV DNA in Cell Lines:

High molecular weight bands of 8-21 kb in Southern blots probed with EBV sequences adjacent to the telomeres of the viral genome probably represent joined terminal fragments of EBV episomes (Kitner and Sugden, 1979; Raab-Traub and Flynn, 1986; Katz *et al.*, 1989) (FigureV-1). When the DNA of Raji cells, which is known to contain only episomal forms of EBV, was digested with *Bam*HI and analyzed by Southern blot, a 21-kb band was demonstrated (Figure V-3, Lanes A to C). The higher molecular weight of fused termini in Raji cells is due to the deletion of one of the *Bam*HI sites. The cell line P3HR-1, which releases low levels of EB virions, contained a faint 12.5-kb fragment (Figure V-3,

Lane D). Tumor promoters, such as *n*-butyrate and TPA, induce the replication of EBV. Both the Raji and P3HR-1 cell lines produced increased levels of EBV DNA after exposure of the cells to TPA (Figure V-3, Lanes C and E). Linear forms of the EBV genome were not observed after induction, perhaps due to the low amount of DNA run in the gels. The cell line B95-8, which is permissive for replication of EBV, demonstrated bands of 10.5, 9.0, and 8.0 kb, and a ladder of at least 5 low molecular weight bands was identified (Figure V-3, Lane F). In contrast, the Southern blot of DNA extracted from purified virions did not show high molecular weight bands, but only a ladder of low molecular weight bands representing linear molecules found in the viral genome (Figure V-3, Lane G).

In order to prove that the observed low molecular weight ladder represented linear EBV molecules, the EBV producer cell line B95-8 was treated with TPA to induce the replication of linear forms of EBV DNA (Figure V-4, Lanes B to D). After treatment of the cells with TPA and ACV, there was inhibition of low molecular weight bands, with some effect seen on the high molecular weight fragments (Figure V-4, Lanes E to G). We also treated the purified cellular DNA and virion DNA with the exonuclease *Bal* 31, which cleaves the free ends of DNA molecules. After digestion with *Bal* 31, there was sequential cleavage and disappearance of the ladder of EBV DNA bands. This is shown in Figure V-5. The linear nature of the DNA extracted from virions was also confirmed (Figure V-5). Lane A shows a sample of purified virion DNA digested with *Bam*HI. Lanes B and C show virion-purified DNA digested with the exonuclease *Bal* 31 prior to digestion with *Bam*HI. Lanes D to G show the Southern blot of DNA extracted from oropharyngeal cells digested with *Bam*HI and probed with the 4.0-kb unique sequence from the left-hand end of the EBV genome. If the DNA extracted was digested with the exonuclease *Bal* 31 prior to digestion with *Bam*HI, this linear

pattern of DNA fragments disappeared (Figure V-5, Lanes E and G). The complete disappearance of the stepladder pattern shown in Lane G after digestion with *Bal* 31 suggests that all forms of virion DNA are linear.

The Configuration of EBV DNA in Patients Shedding High Levels of the Virus:

Eight patients from the more than 100 patients in the Infectious Diseases Clinic referred for investigation of severe headaches and disabling fatigue were identified as high virus shedders. In addition, 17 cardiac transplant patients were studied, and 16 of these were high virus shedders. Samples of DNA extracted from oropharyngeal cells and digested with *Bam*HI are shown in FigureV-6. In the cardiac transplant patients, 9 had both linear and episomal and/or concatemeric forms, and 7 had only linear forms. Examples of specimens from these patients are shown in Figure V-6, Lanes A, C, D, F, G, I, J, L, and M. Lanes A, C, D, F, G, I, and L show examples of forms containing joined termini and linear forms.

The Configuration of EBV DNA in Oropharyngeal Cells of Patients with HIV Infection:

We previously demonstrated that some HIV-seropositive individuals excrete high levels of EBV in the oropharynx and that these individuals are more likely to progress to AIDS. An analysis of the viral DNA produced in the oropharynx of these patients was performed to determine whether the virus was in a replicative or latent form (Figures V-5D and V-5F, and Figures V-7B to V-7F).

Of 25 oropharyngeal cell samples from 23 HIV-seropositive patients, 9 had multiple bands representing linear forms of the EBV genome, 9 had both linear and episomal forms, 1 had only episomal forms, and 1 had a smear and

could not be clearly analyzed. Three patients were negative for EBV DNA. Despite AZT therapy, most patients continued to excrete high levels of EBV, and replication of linear forms of EBV was not inhibited by this therapy. Examples of the analysis of EBV DNA from these patients is shown in Figure V-7, Lanes B to F.

EBV DNA In Patients with Lymphoproliferative Syndrome:

One of the 17 cardiac transplant recipients developed a lymphoproliferative disorder (LPD) after acquiring a primary EBV infection which was characterized by fever, sore throat, and lymphadenopathy. Histologically, a lymph node biopsy demonstrated invasion of vessels and muscle by atypical lymphocytes. This patient excreted three nanograms of EBV DNA per microgram of cellular DNA, which represents a very high level of EBV excretion. Analysis by Southern blot demonstrated both linear forms and forms containing joined termini of the EBV genome in DNA extracted from oropharyngeal cells (Figure V-7G), but only forms with joined termini were demonstrated in tissue from the lymph node (FigureV-7H); however, the signal from the lymph node biopsy was too weak to exclude linear forms. The lymphoproliferative disorder resolved after reducing the dosage of immunosuppressive agents and the administration of IV acyclovir for two weeks.

Discussion

To identify patients at risk of developing EBV-associated disorders early during immunosuppression, it may be useful to measure the levels of EBV replication in

oropharyngeal cells in a prospective manner (see Chapters IV and VI). In this study we have analyzed the replicative forms of EBV DNA in oropharyngeal cells.

Although little is known about the control of viral replication in oropharyngeal cells, EBV infection of the epithelium is probably controlled by the cellular immune response to the virus which includes the production of interferon, the induction of virus-specific cytotoxic T cells, and the secretion of a B cell factor secreted by T cells that down-regulates episomal replication (Morgan *et al.*, 1989). Due to the close association between cell differentiation and EBV replication (Sixbey *et al.*, 1983; Allday and Crawford, 1988), it is likely that other factors, such as the rate of maturation and shedding of the epithelium in the oropharynx, may be involved in determining the levels of EBV replication and excretion in the oropharynx.

At the time this study was begun, there was no published evidence on the nature of the replicative forms of EBV *in vivo*, and it was not known whether mature oropharyngeal cells contained predominantly episomal or linear forms of EBV. It was assumed that immature cells in the oropharynx could maintain the episomal form of EBV since only EBNA is detected in these cells and EBNA is necessary to maintain the episomal state of the virus. Sixbey (1985) demonstrated the presence of EBNA but not of EA or VCA in immature oropharyngeal cells. However, as cells mature, they become permissive for virus replication, with resulting virus release and cell death. It may be that there is a pool of episomal forms from which the linear forms are generated, resulting in the formation of unit size linear molecules. During latent infections, episomal forms of EBV are replicated during the S-1 phase of the cell cycle by cellular DNA polymerases

(Sixbey and Pagano, 1985), which are not affected by antiviral durgs. In contrast, during the lytic cycle replication of linear forms of EBV depends upon the viral DNA polymerase, which may be inhibited by antiviral therapy (Colby *et al.*, 1980; Katz *et al.*, 1989).

Hairy cell leukoplakia is an EBV-related epithelial lesion that occurs in immunosuppressed individuals and is characterized by proliferation of epithelial cells causing the formation of white plaques on the lateral aspect of the tongue. The presence of hairy leukoplakia in asymptomatic HIV-seropositive individuals often predates the onset of AIDS by several months (Resnick *et al.*, 1988). Southern blot analysis of EBNA extracted from these lesions demonstrated the presence of linear forms of the EBV genome, which suggested active replication of the virus in these epithelial lesions (Greenspan *et al.*, 1985).

Recent studies suggest that the analysis of the configuration of the ends of the EBV genome could define clonotype and whether a genome is circular or linear (Raab-Traub and Flynn, 1986; Brown *et al.*, 1988, Katz *et al.*, 1989). Raab-Traub and Flynn (1986) demonstrated episomal forms of EBV in nasopharyngeal carcinoma and suggested that the analysis of the fused termini of the EBV genome defined the clonotype of nasopharyngeal carcinomas and EBV-associated lymphoproliferative disorders. A preponderance of episomal forms of the viral genome was found in lymphocytes (Brown *et al.*, 1988), and episomal and linear forms were detectable in some cases of lymphoproliferative disorders (Katz *et al.*, 1989). The results from our study suggest that a preponderance of linear EBV genomes is found in mature oropharyngeal cells. High levels of genomic forms of EBV that contain joined termini were detected in the

oropharyngeal cells of some immunosuppressed patients. These can represent circular or concatemeric forms of the virus. Under conditions of severe immunosuppression, there is a good possibility that these joined termini represent concatemeric, rather than episomal forms of the viral genome. The analysis of the viral DNA from oropharyngeal cells indicated that some individuals were infected with forms of EBV that contained only one type of fused termini, yet we found no evidence of monoclonal proliferation in these individuals. This would raise some doubt about the use of this technique to define clonal types in EBV-associated tumors.

The level of EBV excretion is an important marker, and possibly a co-factor, in the progression of disease in HIV-seropositive individuals (Diaz-Mitoma *et al.*, 1990, Chapter IV). Although the increased EBV load observed in progressive HIV infection may be related to low T4 lymphocyte subsets and a decrease in the production of IL-2 (Blumberg *et al.*, 1987), it is important to determine whether EBV has an effect on the progression of HIV infection. In HIV-seropositive individuals with high EBV excretion, the administration of EBV-specific antiviral agents in combination with anti-HIV therapy in order to decrease the virus load could determine whether an individual develops EBV-associated disorders such as hairy leukoplakia or lymphomas. Antiviral therapy directed against EBV may also slow the progression of HIV-associated disease. The preliminary results of a clinical trial of high-dose acycloguanosine and AZT suggest that progressive HIV infection may be inhibited by this approach (Fiddian *et al.*, 1989). Although AZT has been reported to inhibit EBV *in vitro* (Lin *et al.*, 1989), most HIV-seropositive individuals in our study had replicating forms of the EBV in oropharyngeal cells despite AZT therapy.

Antiviral agents such as acycloguanosine have been tried in the treatment of EBV-associated disorders. For instance, a placebo-controlled clinical trial evaluated acycloguanosine therapy for IM (Andersson *et al.*, 1986). Attempts have also been made to treat EBV-associated lymphoproliferative disorders with antiviral therapy (Hanto *et al.*, 1982); however, the effects of antiviral therapy in IM or in lymphoproliferative disorders have been marginal or disappointing. Perhaps the unimpressive results obtained in these trials were due to the late administration of antiviral therapy. Symptomatic infectious mononucleosis occurs late, after virus replication in oropharyngeal cells and invasion of B cells. Most of the disease manifestations in infectious mononucleosis are a consequence of the immune response to the EBV infection of B lymphocytes and oropharyngeal epithelial cells. The unsuccessful attempts to treat LPD with acyclovir may also be due to a late institution of therapy when there is already a large virus load causing polyclonal activation of infected B cells. EBV infection of B cells is not susceptible to antiviral therapy.

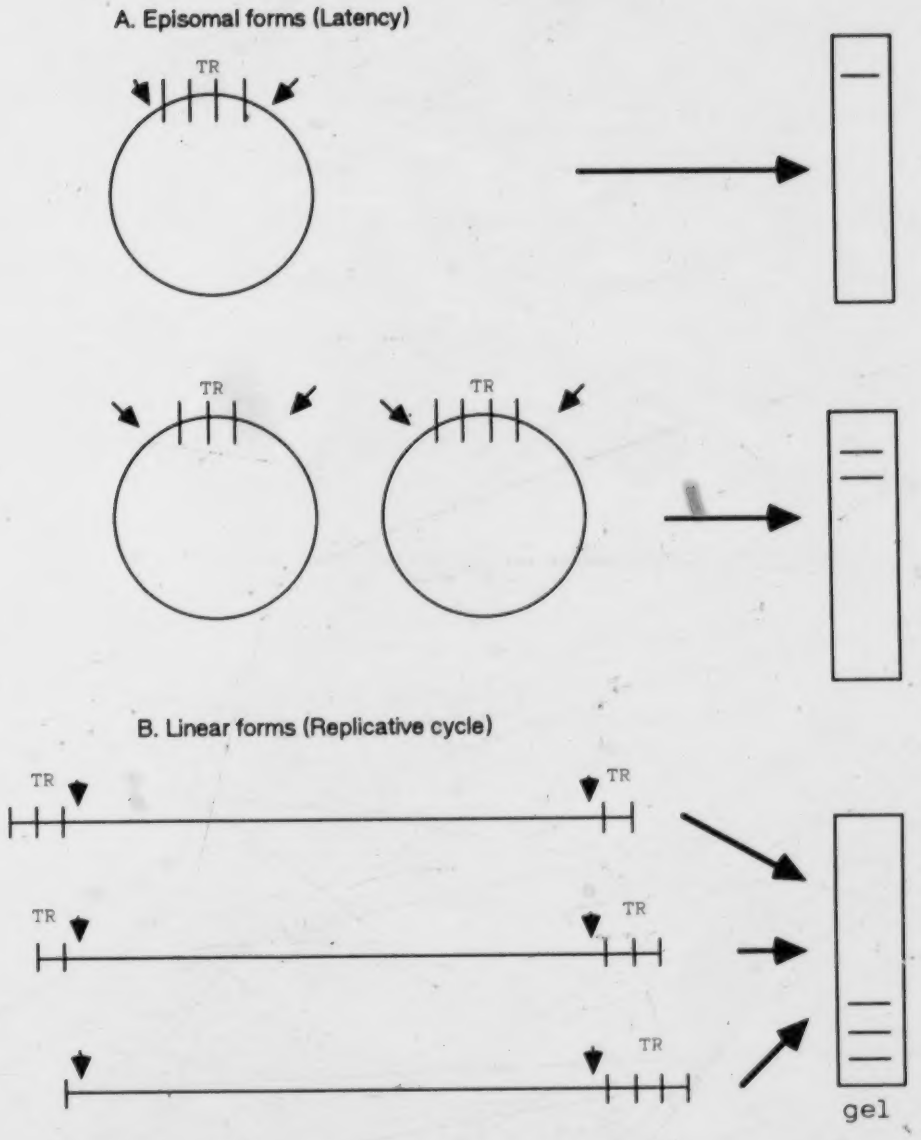

Figure V-1. Molecular configuration of the EBV genome. Episomal or concatemeric forms (A) contain joined termini and have higher MW than the free ends of linear molecules (B). The expected bands in Southern blots of viral DNA digested with Bam HI (▼) and probed with radiolabeled-subgenomic EBV fragments adjacent to the TRs are shown on the right side of the figure.

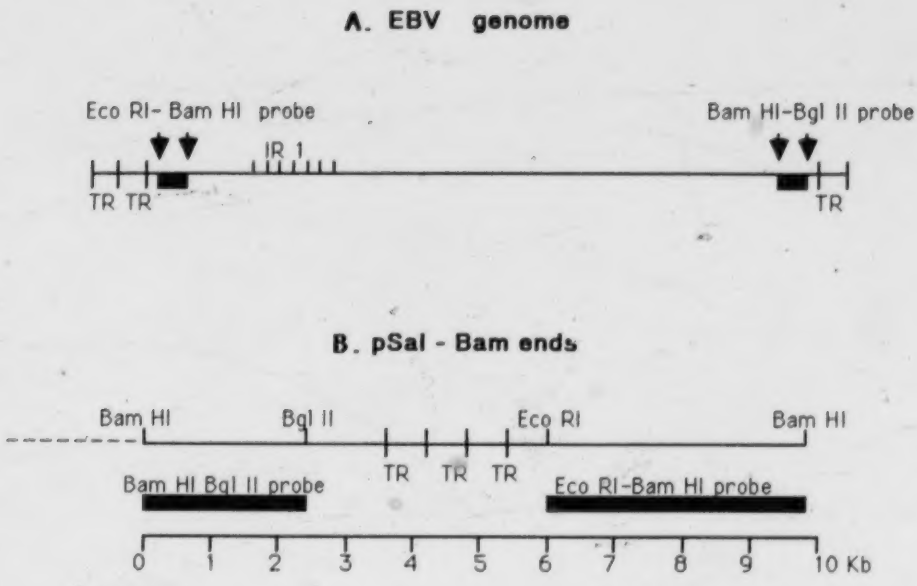

Figure V-2. Location of the EBV subgenomic fragments used as DNA probes in the detection of joined or free termini of the EBV genome. (A) genomic map of EBV and restriction endonuclease cleavage sites delimiting the location of the probes. (B) map of the pSal-Bam ends plasmid, from which the probes were isolated.

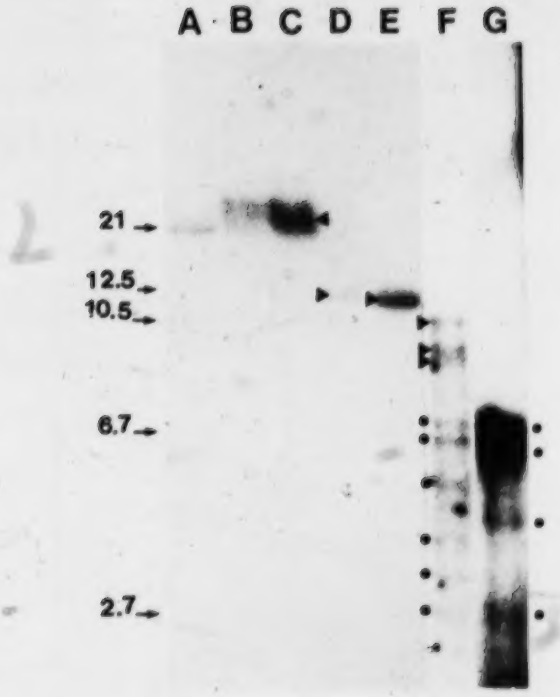

Figure V-3. Southern blot probed with the 4.0 kb EcoRI-*Bam*HI EBV subgenomic fragment. The DNAs in this filter were digested with *Bam*HI. Lanes *A*, *B*, and *C* contain one microgram of DNA from Raji cells. *(A)* Controls. *(B)* Cells treated with *n*-butyrate for 24 hrs. *(C)* Cells treated with *n*-butyrate and TPA for 24 hrs. Lanes D and E contain one microgram of DNA from P3HR-I cells. *(D)* Controls. *(E)* Cells treated with TPA for 24 hrs. *(F)* One microgram of DNA from the B95-8 cell line. *(G)* EBV DNA extracted from virions (B95-8).

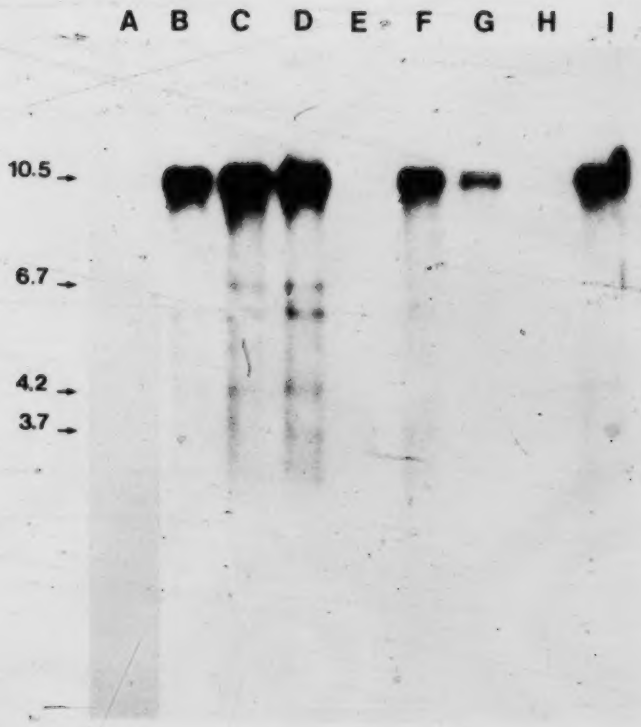

Figure V-4. Treatment of the cell line B95-8 with acycloguanosine to demonstrate that the bands with a stepladder pattern represent linear forms of the EBV genome. Approximately 1 microgram of DNA was digested with *Bam*HI and was run in an agarose gel. Southern blots were hybridized with the radiolabeled Bgl II-*Bam*HI (2.4 kb) probe. *(A)* 18-hour control. *(B, C, and D)* Cells treated with TPA (4.0 ng/ml) for 18 hours, 28 hours, and 72 hours, respectively. *(E, F, and G)* (in G, well leaked and DNA was partially lost). Cells treated with TPA (40 ng/ml) and acycloguanosine (100 μM) for 18 hours, 28 hours, and 72 hours, respectively. *(H and I)* Cells treated with acycloguanosine only, for 18 hours and 72 hours, respectively.

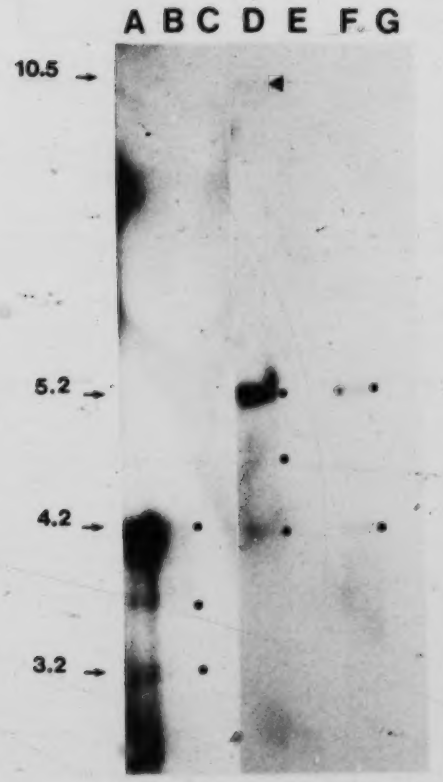

Figure V-5. Experiment to demonstrate digestion of the free ends of the EBV genome by the exonuclease *Bal* 31. The filter was probed with the 4 kb EcoRI-*Bam*HI EBV subgenomic fragment, which is adjacent to the left end of the EBV genome. *(A, B, and C)* Samples contain 20 picograms of EBV DNA extracted from virions. *(A)* DNA digested with *Bam*HI only. *(B)* DNA digested with *Bal* 31 for 15 minutes, followed by digestion with *Bam*HI. *(C)* DNA treated with *Bal* 31 for 45 minutes, followed by digestion with *Bam*HI. *(D)* DNA (1.5 micrograms) extracted from oropharyngeal cells from a patient with AIDS. The DNA was digested with *Bam*HI only. *(E)* DNA (1.5 micrograms) from the same patient described in *D*. The DNA was first digested with *Bal* 31 for 10 minutes, followed by digestion with *Bam*HI. *(F and G)* DNA (1.5 micrograms) extracted from oropharyngeal cells from a patient with AIDS. The DNA in *G* was digested with *Bam*HI only, and in *D* was digested with *Bal* 31 for 10 minutes, followed by digestion with *Bam*HI.

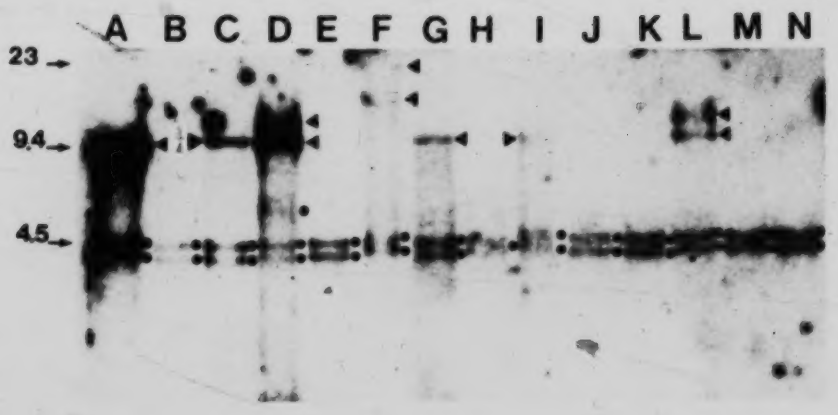

Figure V-6. EBV telomeric analysis in oropharyngeal cells of cardiac transplant recipients. The Southern blot was probed with the 2.4 kb *Bam*HI-Bgl II EBV subgenomic fragment, which is adjacent to the right end of the EBV genome. All DNAs were digested with *Bam*HI. *(A, C, D, F, G, I, J, L,* and *M)* DNAs from cardiac transplant recipients on cyclosporin and prednisone. *(B, E, H, K,* and *N)* DNAs from EBV-seropositive individuals referred because of chronic fatigue or headaches.

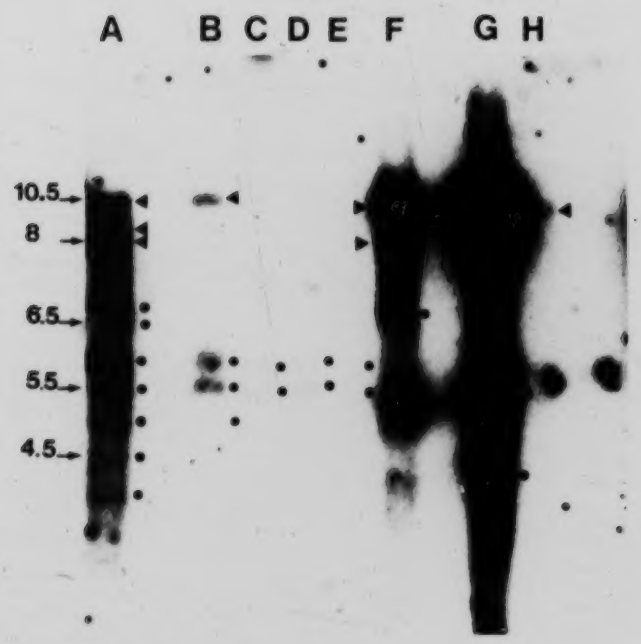

Figure V-7. EBV telomere analysis in human tissues. Southern blot probed with the 4 kb EcoRI-*Bam*HI EBV telomeric fragment. All DNAs were treated with *Bam*HI. *(A)* B95-8 DNA (1 microgram). *(B, C, D, E,* and *F)* Oropharyngeal cellular DNA from patients with AIDS on AZT therapy. *(G)* DNA from oropharyngeal cells from a cardiac transplant recipient who developed lymphoproliferative disease. *(H)* DNA from a lymph node biopsy from the patient described in *G*

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CHAPTER VI

HIGH LEVELS OF VIRAL REPLICATION OF TYPE A VARIANTS OF EPSTEIN-BARR VIRUS ARE INVOLVED IN THE PATHOGENESIS OF LYMPHOPROLIFERATIVE DISORDERS IN ORGAN TRANSPLANT PATIENTS

A VERSION OF THIS CHAPTER IS IN PREPARATION FOR PUBLICATION as, "High levels of viral replication of type A variants of Epstein-Barr virus are involved in the pathogenesis of lymphoproliferative disorders in organ transplant patients." F. Diaz-Mitoma, A. Ruiz, J.K. Preiksaitis, D.L.J. Tyrrell.

Introduction

Organ transplantation is a frequent form of treatment for end-stage organ failure. Immunosuppressive agents are necessary to avoid rejection of the allograft; however, long-term immunosuppression places recipients at increased risk of infection and the development of neoplastic lesions such as skin cancer and lymphoproliferative disorders (LPDs) (Frizzera *et al.*, 1981; Cleary *et al.*, 1986).

Ho *et al.* (1985) demonstrated that post-transplant LPDs are more frequently associated with primary EBV infection than reactivation; however, LPDs are also observed in patients who were EBV-seropositive before transplantation. The abundance of replicative forms of EBV in the oropharynx of immunosuppressed patients (Chapter V) may account for the high level of B cell infection by EBV in the peripheral circulation (Yao *et al.*, 1985). B lymphocytes may be infected during episodes of viremia (Ablashi *et* al., 1987) or when they pass through Waldeyer's ring and are in close contact with the oropharyngeal epithelium. The pathogenesis of LPDs may involve a dual process of an increase in EBV replication with subsequent infection of B cells, and reduced immunosurveillance which could allow for invasion of tissues by proliferating B cells.

There are two EBV variants (as discussed in Chapter I) which are distinguished by their divergency in the EBNA-2 gene (Zimber *et al.*, 1986; Young *et al.*, 1987). The EBNA-2 protein is responsible for the transforming phenotype of the EBV variants. Type A variants of EBV have a higher transforming efficiency than do type B variants (Rickinson *et al.*, 1987). Sixbey *et al.* (1989) analyzed the

prevalence of type A and type B variants in Memphis, Tennessee, and found that 50% of healthy EBV excreters were infected with type A, 41% were infected with type B, and 9% were infected with both variants. We believe that, because of their transforming ability, type A strain variants could be involved more frequently or even exclusively in the pathogenesis of the LPDs. In order to assess whether increased levels of EBV replication precede the development of LPDs, we prospectively determined the virus load in transplant patients by measuring the levels of oropharyngeal EBV DNA. We also established whether type A or type B variants were more commonly associated with the development of LPDs.

Materials and Methods

Study Patients:

Subjects for this study included 6 patients who developed lymphoproliferative disorders following organ transplantation (4 received hearts and 2 received kidneys) and 6 sex- and organ-matched controls. These patients were chosen from a population of 58 cardiac and 142 renal allograft recipients who had been prospectively followed by Dr. Jutta K. Preiksaitis. Patients were asked to gargle with 15 ml of virus-transport medium without antibiotics once or twice before transplantation and every 2-3 weeks after transplantation for variable periods of time. The subjects of this study were followed for a median of 32 weeks (range: 8-30 weeks). Blood specimens were also drawn for viral serology at the same time as throat washings were obtained. Throat washing specimens were stored at -70°C in 10% DMSO until DNA extraction for the detection of EBV.

Conventional histopathologic criteria were used to make a diagnosis of LPD (Randhawa *et al.*, 1989).

EBV serology was performed before and 4-8 weeks after transplantation. ELISAs were performed to detect IgG antibodies to EA, VCA, and EBNA, as previously described (Luka *et al.*, 1984) using commerical kits (Dupont Co., Billeria, Mass.).

Surface Marker Studies:

Tissues from the 6 patients with LPDs were available for surface marker analysis. Monoclonal antibodies against the following lymphocyte surface proteins were used for the immunofluorescent assays: CD2, CD3, CD20, CD22, CD43, CD21 (C3D receptor), CD30; the alpha, delta, gamma, kappa, lambda, and mu chains; and T4 and T8 molecules. These studies were done by Dr. S. Poppema at the Cross Cancer Institute (Edmonton, Alberta).

Quantitation of EBV DNA:

A modification of the method described in Chapter IV was used to quantitate the levels of EBV DNA in the oropharynx (Diaz-Mitoma *et al.*, 1990). DNA was extracted from oropharyngeal cells (Meinkoth and Wahl, 1984) and spotted onto nylon filters. The filters were prehybridized and hybridized as described in Chapter V, the only difference being the lack of dextran sulfate in the hybridization solution. Posthybridization washings were performed at room temperature twice in 2 x SSC and once in 0.1 x SSC and 0.1% SDS at 65°C for 1 hr. Filters were probed with a pool of purified EBV subgenomic fragments, including the *Bam*HI fragment M (3.0 kb) and the fragments *Bam*HI-EcoR1 (4.0 kb) and Bgl II-*Bam*HI (2.4 kb) which are unique regions adjacent to the left and

right terminal fragments of the EBV telomeres, respectively. The filters were first probed with the *Bam*HI-W to ensure a high specificity and sensitivity in the detections of EBV DNA (Chapter II). The probes were isolated from the recombinant plasmids after restriction endonuclease digestion and electrophoresis in low melting point agarose gels, followed by extraction of the DNA (Sambrook *et al.*, 1989). The probes were labeled with (^{32}P)-dCTP by nick translation (Amersham nick translation kit) to a specific activity of >10^8 cpm/μg DNA. The quantitation of EBV DNA was performed as described in Chapter IV, except that in this case Raji cell DNA was used in place of B95-8 cell DNA. Raji cells contain approximately 60 copies of the EBV genome per cell (Andersson and Lindahl, 1976).

The amount of human DNA spotted in each dot was determined by probing the filters with an actin gene, followed by overnight autoradiography of the filters with intensifying screens. The dots were analyzed by densitometry scanning using a model 30 chromoscan densitometer (Joyce-Loebl). A serial dilution of placental DNA was used to develop the standard curve for human DNA using densitometry scanning of the dots generated with the actin gene probe.

Polymerase Chain Reaction (PCR):

Aliquots of approximately 0.05-0.1 micrograms of human DNA extracted from oropharyngeal cells were obtained from samples used in the dot hybridization. These aliquots were used for amplification of EBV DNA sequences by the PCR. Human DNA from the EBV-negative cell line Ramos and DNA extracted from a human lung fibroblast cell line (T_2) infected with HSV were used as negative controls. DNA extracted from the EBV-positive cell line Raji and the

cell line B95-8, which carry type A EBV variants, and the cell lines AG876 and BL16, which carry type B EBV variants, were used as positive controls.

For the PCR analysis we followed the method of Sixbey *et al.* (1989) in which a pair of oligonucleotide primers complementary to conserved regions in both EBNA-2A (type A) and EBNA-2B (type B) genes are used to amplify a region that has a 50% nucleotide sequence homology between the type A and type B variants. A DNA synthesizer "PCR-MATE" (Applied Biosystems, Santa Barbara, Calif.) was used for the synthesis of genotype-specific oligonucleotide probes (18 mer) according to known nucleotide sequences from the prototype EBV variants for EBNA-2A (B95-8) and EBNA-2B (AG876) (Dambaugh *et al.*, 1984). Figure VI-1 shows the 89-base-long sequence used for amplification of a region of the EBNA-2 gene and the genotype-specific oligonucleotide probes that identify the type A and type B variants of the EBV.

The 5' ends of the oligonucleotide probes were labeled with T4 polynucleotide kinase (Pharmacia) (Sambrook *et al.*, 1989). 20 p moles of probe were incubated at 37°C for 60 mins in 0.05 *M tris*-HCl (pH 7.6), 0.01 *M* $MgCl_2$, 0.005 *M* dithiotritol, 0.0001 *M* spermidine HCl, and 0.0001 *M* EDTA, 50 µCi of $\gamma(^{32}P)$-ATP, and 8 units of T4 polynucleotide kinase. The 89-base-pair EBNA-2 was amplified with *Taq* polymerase (Cetus Corporation, Emerville, Calif.) in a 100 µl reaction containing 5 µl of the sample DNA, 10 µl of 0.2 *M tris*-HCl (pH 8.3), 1 µl of 0.2 *M* $MgCl_2$, 10 µl of 0.25 *M* KCl, 1 µl of 5% Tween 20, 5 µl of 1% gelatin solution, 2 µl of 2.5 mM d•NTPs, 40 p moles each of primer 1 and primer 2, 0.5 µl of *Taq* polymerase (2.5 units), and distilled H_2O to make 100 µl of total volume. 75 µl of light mineral oil was layered on top of the reaction. The tubes were incubated in a thermal cycler (Perkin Elmer Cetus, Emerville, Calif.) for 30 cycles

of amplification which consisted of 60 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 70°C. The third step of the last cycle was held at 70°C for 5 mins.

20 µl of the amplified reaction was electrophoresed in 1.5% agarose gels (Bethesda Research Laboratories, Bethesda, Maryland) at 50 V for 3 hrs before dotting the DNA onto nylon filters (Amersham, Oakville, Ont.). The filters were prehybridized for 4 hrs in 6 x SSC, 5 x Denhardt's solution with 0.5 mg/ml sheared and denatured salmon DNA. Hybridization was performed in 10 ml of hybridization solution containing 10^6 cpm/ml of the end-labeled probes at 44°C for the type A probe and at 51°C for the type B probe. Posthybridization washings were carried out at the hybridization temperature for 45 mins in 6 x SSC and 0.1% SDS. Filters were exposed to Kodak X-OMAT AR5 film (Kodak, Rochester, N.Y.) in intensifying screens at -70°C for 5-12 hours.

Results

A total of 142 cadaveric kidney and 58 heart transplants were performed between January 1987 and December 1989. A diagnosis of LPD was made in six cases: two in cadaveric kidney transplants, giving a prevalence of 1.4% in this population, and four cases in heart transplants, giving a prevalence of 6.9% in this second population. The median age of patients with LPDs was 36 years (range: 13-64) and 46 years for the controls (range: 36-56). There were 4 males and 2 females in each group. The median time for development of LPDs was 12 weeks (range: 6-26 weeks) from the date of transplantation. Three of 6 patients with LPDs were EBV-seronegative before transplantation. These 3 patients received allografts from EBV-seropositive donors. All three EBV-seronegative

individuals developed LPDs within 6-8 weeks, the expected period of incubation for acute infectious mononucleosis in adults. All patients and controls received conventional immunosuppressive regimens. All 12 patients were on oral prednisone, cyclosporin A, and azathioprine (imuran). All patients received a murine monoclonal antibody against human T cells (anti-OKT3) for a median time of 16.5 days (range: 7-29). Two patients who developed LPDs also received equine anti-human thymocyte globulin. The surface marker analyses in the lymphoproliferative tissues demonstrated B cell polyclonal proliferations in 4 patients. Two patients had uninterpretable results. Three of the 6 patients with LPDs died.

In order to quantitate the human DNA (hDNA) extracted from oropharyngeal cells, filters were hybridized with the (^{32}P)-labeled actin gene. An example of a filter hybridized with the (^{32}P)-labeled actin gene is shown in Figure VI-2. The same filters, but probed with the radiolabeled *Bam*HI-W subgenomic fragments are shown in Figure VI-3. In order to quantitate the amount of EBV DNA, filters were also probed with the radiolabeled EBV subgenomic fragments. For comparison, in Figure VI-4, rows 1, 2, 3, and 4, represent DNA from allograft recipients with LPDs, and rows 5, 6, and 7 are dots from control patients.

All individuals had negative dot blots for EBV before transplantation. However, post-transplantation, all patients (LPD patients and controls) demonstrated increasing levels of EBV DNA after the transplant, coinciding with episodes of immunosuppression. The peak levels of EBV excretion were higher in patients with LPDs than in controls [median: 1.333 ng (range: 0.335-3.187) vs. median: 0.088 ng (range: 0.007-0.200) EBV DNA/μg hDNA; F = 11.099 (ANOVA); $p \leq$

0.005) (Figure VI-5). The peaks of EBV excretion occurred within a median time of 6 weeks after the transplant (range: 3-16 weeks).

Figures VI-6 to VI-10 show the levels of EBV excretion following surgery in 5 patients developing LPD. These figures also show the results of the PCR analysis of the genotype of the EBNA-2 gene as well as the correlation between levels of EBV DNA and the institution of antiviral therapy. High levels of EBV replication preceded the onset of LPD in all patients. Patient No. 6 died one month after transplantation, and there were only 2 specimens available for analysis. LPD was diagnosed ante mortem, but, due to the small number of throat washings, we were unable to correlate the development of LPD with peaks of EBV excretion. There was a median time of 8 weeks (range: 2-10 weeks) from peak EBV excretion to the diagnosis of LPD.

Typing of the EBV by PCR Amplification and by Genotype-Specific Oligonucleotide Probes:

Figure VI-11 shows the analysis of the 89 bp amplified EBNA-2 gene sequence in patients with the diagnosis of LPD. All 6 patients were positive for type A EBV variants. We also analyzed 3 patients with cardiac and 1 patient with renal transplants for the genotype of the EBNA-2 gene and found that 1 of the 4 was infected with type B variant. All seven strains that were available for typing (six strains from LPD patients and one from controls) were type A or B. There were no amplified products which did not hybridize to the genotype-specific oligonucleotides.

Discussion

EBV has been etiologically linked to post-transplant LPDs (Purtilo *et al.*, 1979; Nalesnick *et al.*, 1988). EBV DNA can be demonstrated in lymphoproliferative lesions, and LPDs occur more frequently after a primary EBV infection than during reactivation (Ho *et al.*, 1985; Rhandhawa *et al.*, 1989).

The manifestations of disease caused by LPDs are secondary to rapidly dividing B cells in affected tissues. The lymphoproliferation may be polyclonal or monoclonal, but clonality does not define the aggressiveness of LPDs (Katz *et al.*, 1989). Both polyclonal and monoclonal lymphoproliferative disorders have been associated with a fatal outcome. The treatment of LPDs consists of a reduction of immunosuppression and/or the administration of antiviral therapy. When the LPD is of a monoclonal phenotype, cancer chemotherapy or radiotherapy is required. Although viral serology identifies the subgroup of EBV-seronegative patients who are at high risk of developing LPDs, viral serology does not identify the EBV-seropositive patients at risk of developing LPDs, and it is not an effective way to monitor patients in order to predict the development of disease.

In this study we have quantified the amount of EBV DNA detectable in throat washings. The patients that developed LPDs excreted significantly higher amounts of EBV DNA than organ- and sex-matched controls. The detection of very high levels of EBV DNA in oropharyngeal cells from post-transplantation patients has important predictive value for the development of LPDs. This study suggests that patients who are at risk of developing LPDs can be identified 2-10 weeks before the onset of LPD by the high levels of EBV DNA in the oropharynx

(≤ 0.2 nanograms of EBV DNA/μg DNA). This may have implications for the management of these patients. The institution of antiviral therapy in individuals demonstrating high levels of EBV replication after transplantation may prevent extensive infection of B cells. Such intervention may prevent the development of LPDs.

In this study we have also shown that individuals developing LPDs are more likely to be infected with the type A variant of EBV. This phenotype of EBV depends on unique properties of the EBNA-2 gene to transform B cells. The gene product of EBNA-2 probably acts on B cells by transactivating the CD23 surface protein (Rickinson *et al.*, 1987). There is evidence that this molecule of 90 kDa (a dimer of a 45-kDa protein) functions as a receptor for a B cell growth factor (Gordon *et al.*, 1986). The EBNA-2 gene could be analogous to the *tat* gene of the HIV-2 which transactivates the interleukin 2 receptor gene (Greene *et al.*, 1986).

To determine which genotype of the EBNA-2 gene was prevalent in LPDs, we used polymerase chain reaction amplification to analyze the DNA from oropharyngeal cells. This procedure has the advantage of being able to analyze small amounts of the samples in a single reaction to determine which genotype of EBV was involved in the development of LPDs. Although the numbers were small, our results indicated that EBV type A was strongly associated with the development of LPDs. No patient who developed an LPD had high levels of EBV type B.

The median level of EBV DNA in patients developing LPDs was approximately 15 times higher than that of transplant controls who did not develop LPDs.

In conclusion, this study showed that high EBV DNA excretion is frequently associated with the development of LPDs. In addition, high EBV DNA excretion is of predictive value for the development of an LPD in transplant patients. Finally, in this study type A EBV was associated with development of LPDs, whereas type B EBV was not. However, the number of cases and controls in this study was small, and more cases and controls will have to be followed to confirm this observation.

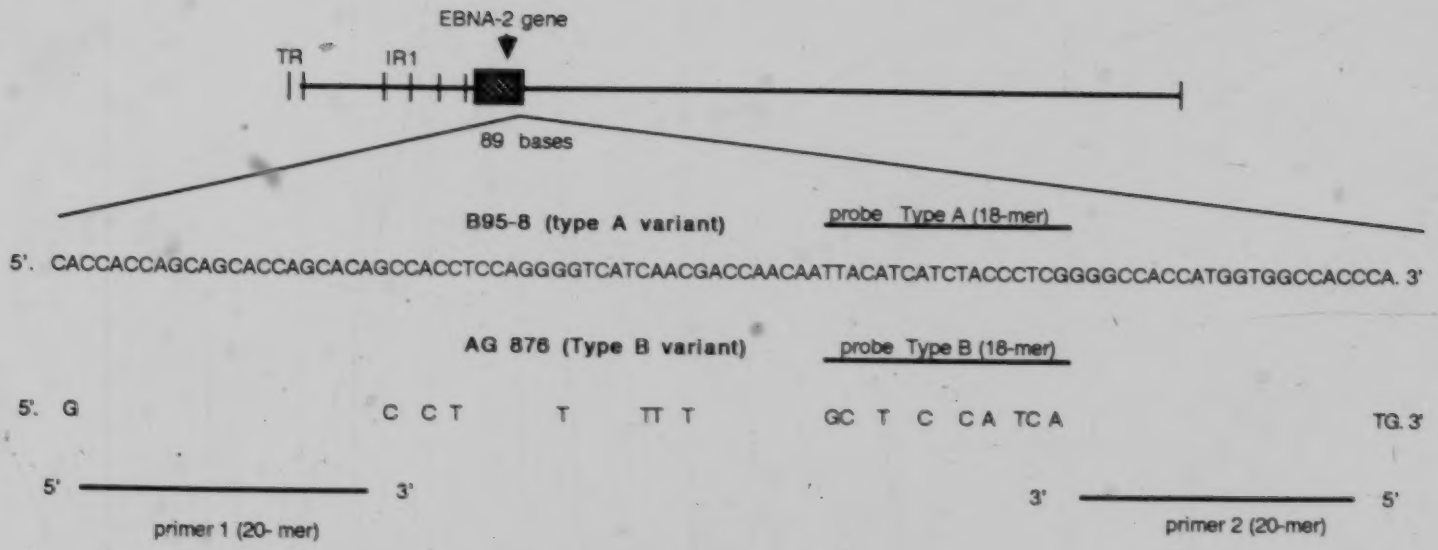

Figure VI-1. Location of the EBNA-2 gene within the EBV genome, and strategy to define the genotype of the EBV by using the polymerase chain reaction.

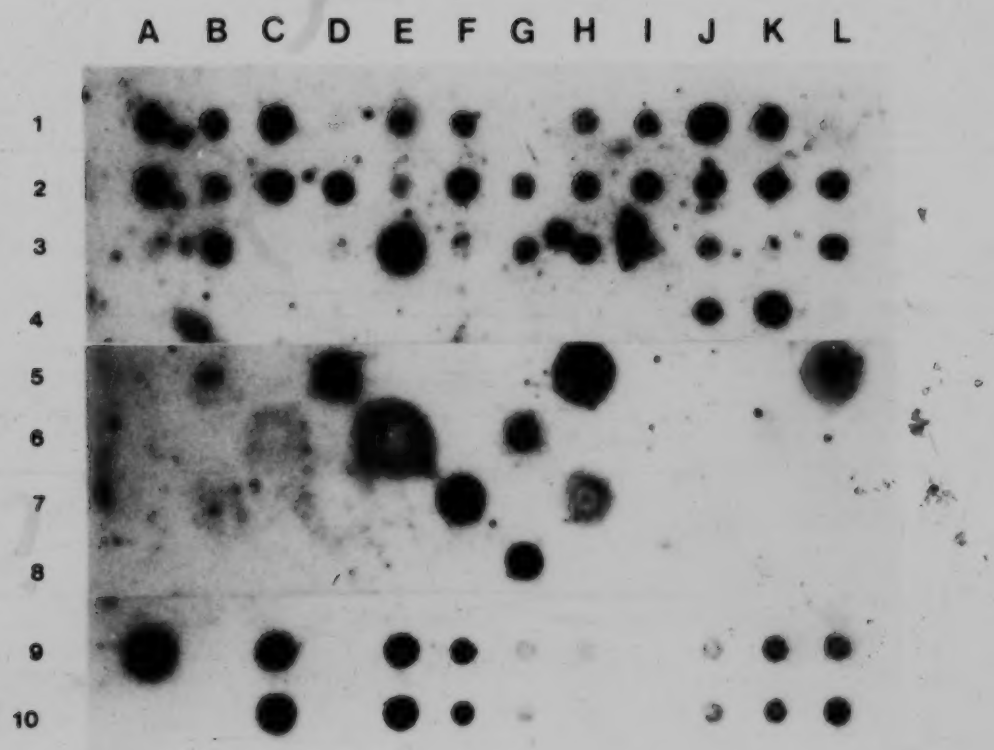

Figure VI-2. Dot-blot to quantitate human DNA extracted from oropharyngeal cells. The filters were hybridized with the ^{32}P-labeled actin gene. Rows 1 to 8 are the dots from the DNAs from control patients. Rows 9 and 10, Columns *A*, *C*, *E*, and *F* are dots containing serial dilutions of DNA from the cell line Raji starting at 0.5 micrograms; Columns *J*, *K*, and *L* are dots that contain 0.02, 0.05, and 0.1 micrograms of human placental DNA, respectively.

Figure VI-3. Specificity of the *Bam*HI-W probe for the detection of EBV DNA. This is the autoradiograph of the same filters as those shown in Figure VI-2, but hybridized with the ^{32}P-labeled *Bam*HI-W subgenomic fragment. Rows 1 to 8 are dots from the DNAs from control patients. Rows 9 and 10, Columns *A, C,* and *E* to *H* are serial dilutions of DNA extracted from Raji cells.

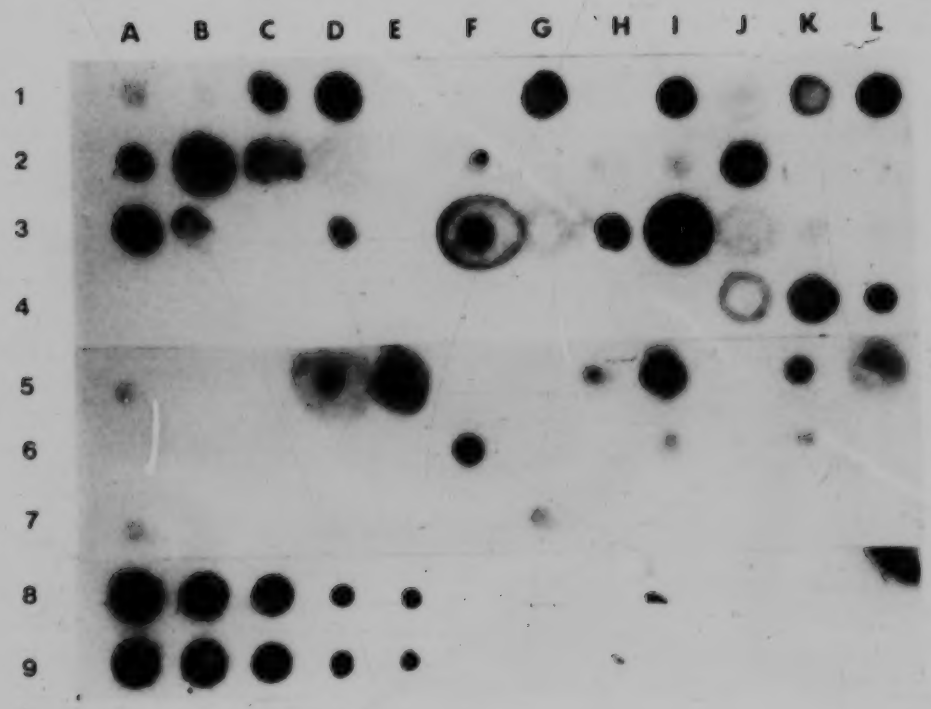

Figure VI-4. Dot-blot to quantitate EBV DNA in oropharyngeal cells. Rows 1 to 4 are dots from patients with lymphoproliferative disorders, and Rows 5 to 7 are from control patients. The approximate amounts of EBV DNA contained in each dot were calculated from the curve drawn from the densitometry values of the serial dilution of DNA extracted from the cell line Raji (Rows 8 and 9, Lanes *A, B, C, D,* and *E* represent 0.1, 0.05, 0.02, 0.01, and 0.005 micrograms of DNA, respectively). Rows 8 and 9, Lanes *H, I, J,* and *K* were spotted with human placental DNA, at concentrations of 3.9, 2.2, 1.1, and 0.6 micrograms, respectively. The samples were studied in a random and blind sequence for LPD and controls. After the results were known, the code was broken and the data used to develop the data shown in Figures VI-5 to VI-10.

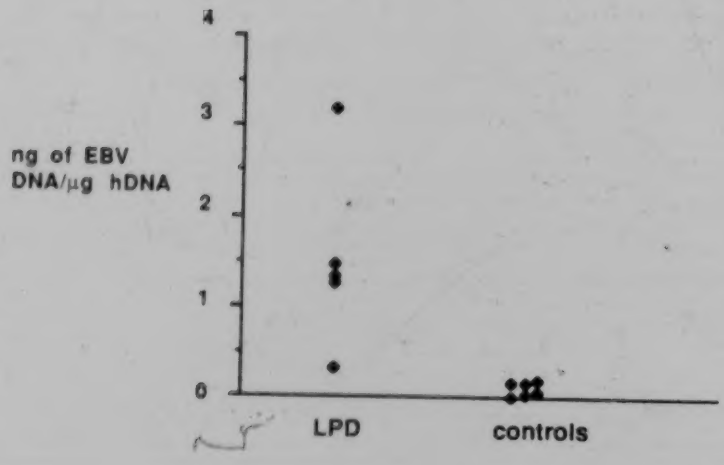

Figure VI-5. Peak levels of EBV DNA in oropharyngeal cells of patients with LPD and in controls. The levels of EBV DNA in patients developing LPD were fifteen times higher than in the control group (medians: 1.33 vs .088 nanograms of EBV DNA/µg of hDNA, respectively. F= 14, [ANOVA], p≤0.005).

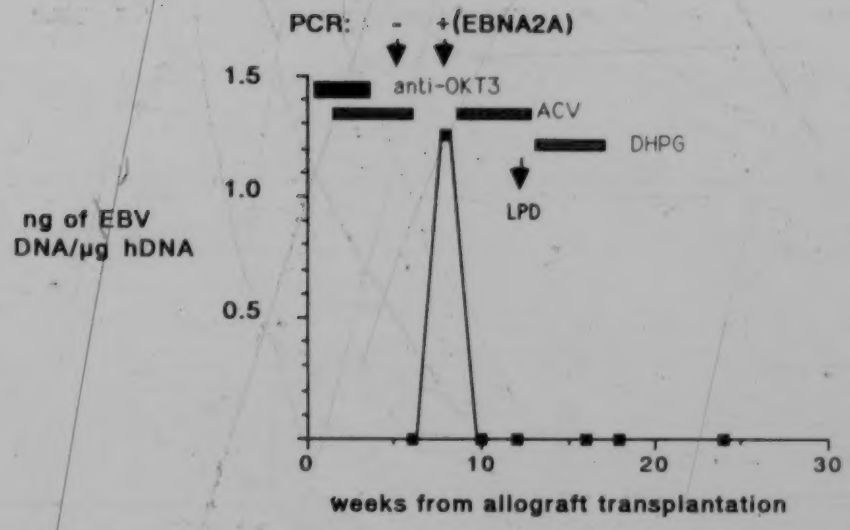

Figure VI-6. Correlation of the levels of EBV DNA and the institution of antiviral therapy post-transplantation. Patient no. 1 was EBV-seropositive before transplantation, a reactivation of infection of a type A variant of the EBV, followed by perforation of the small bowel due to aggressive lymphoproliferation. This patient had a good response to the reduction of the immunosuppressants and administration of acycloguanosine (ACV), and dihydroxy-acycloguanosine (DHPG).

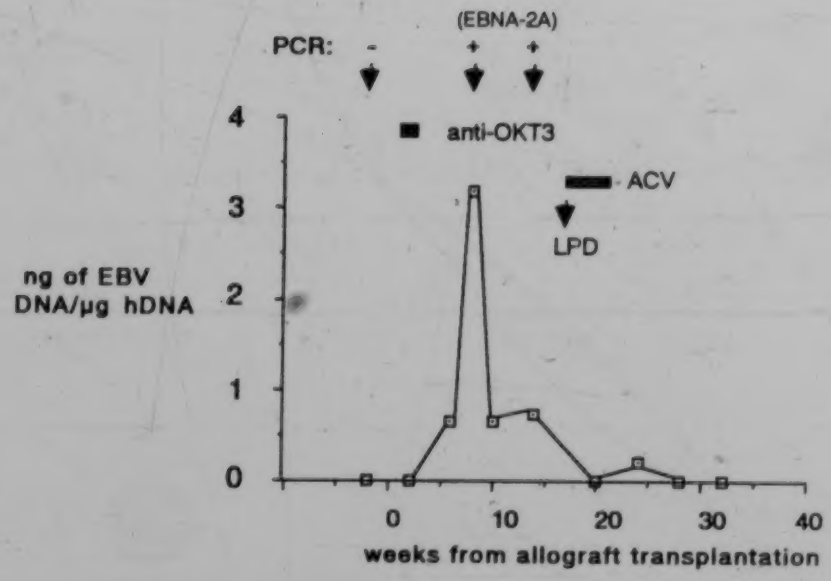

Figure VI-7. Correlation of the levels of EBV DNA with the time of transplantation, and institution of immunosuppressive and antiviral therapy in patient no. 2; a 13 year-old EBV-seronegative patient, who received a heart allograft from an EBV-seropositive donor. High levels of EBV DNA from a type A variant were detected by dot hybridization and polymerase chain reaction amplification approximately 2 weeks before the onset of LPD. She responded favorably to antiviral therapy and reduction of immunosuppression.

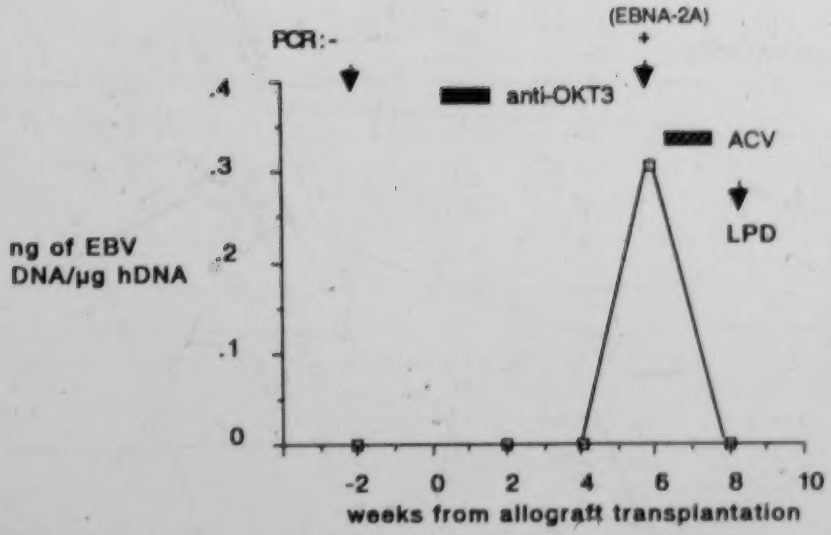

Figure VI-8. Patient no. 3 was a 48 year-old EBV-seropositive female, who developed reactivation of a type A variant of EBV 3 weeks before developing fatal lymphoproliferation associated with encephalopathy and hepatitis.

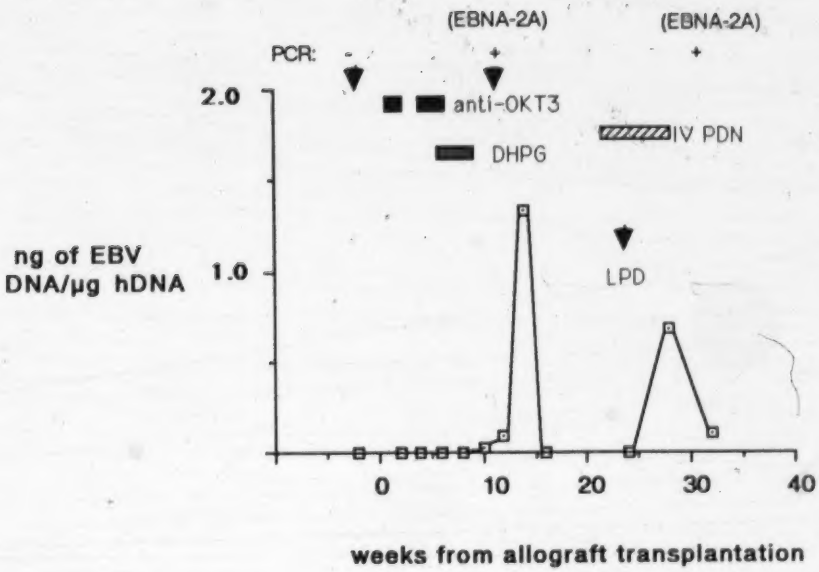

Figure VI-9. Patient no. 4 developed aggressive lymphoproliferation in the small bowel 8 weeks after the detection of high levels of replication of a type A variant of EBV. A second episode of EBV reactivation coincided with the administration of IV prednisolone (PDN)

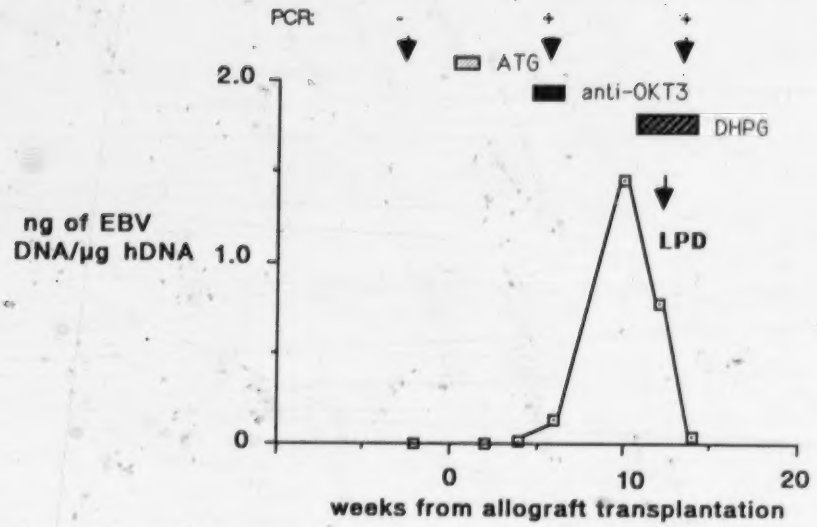

Figure VI-10. Correlation of the institution of immunosuppressive therapy, levels of EBV DNA, and the administration of antiviral agents (DHPG). This 26 year-old EBV-seronegative patient received a kidney transplant from an EBV-seropositive donor, developing aggressive lymphoproliferation, which resulted in fatal cerebritis and liver failure. The lymphoproliferation was caused by a type A variant of EBV.

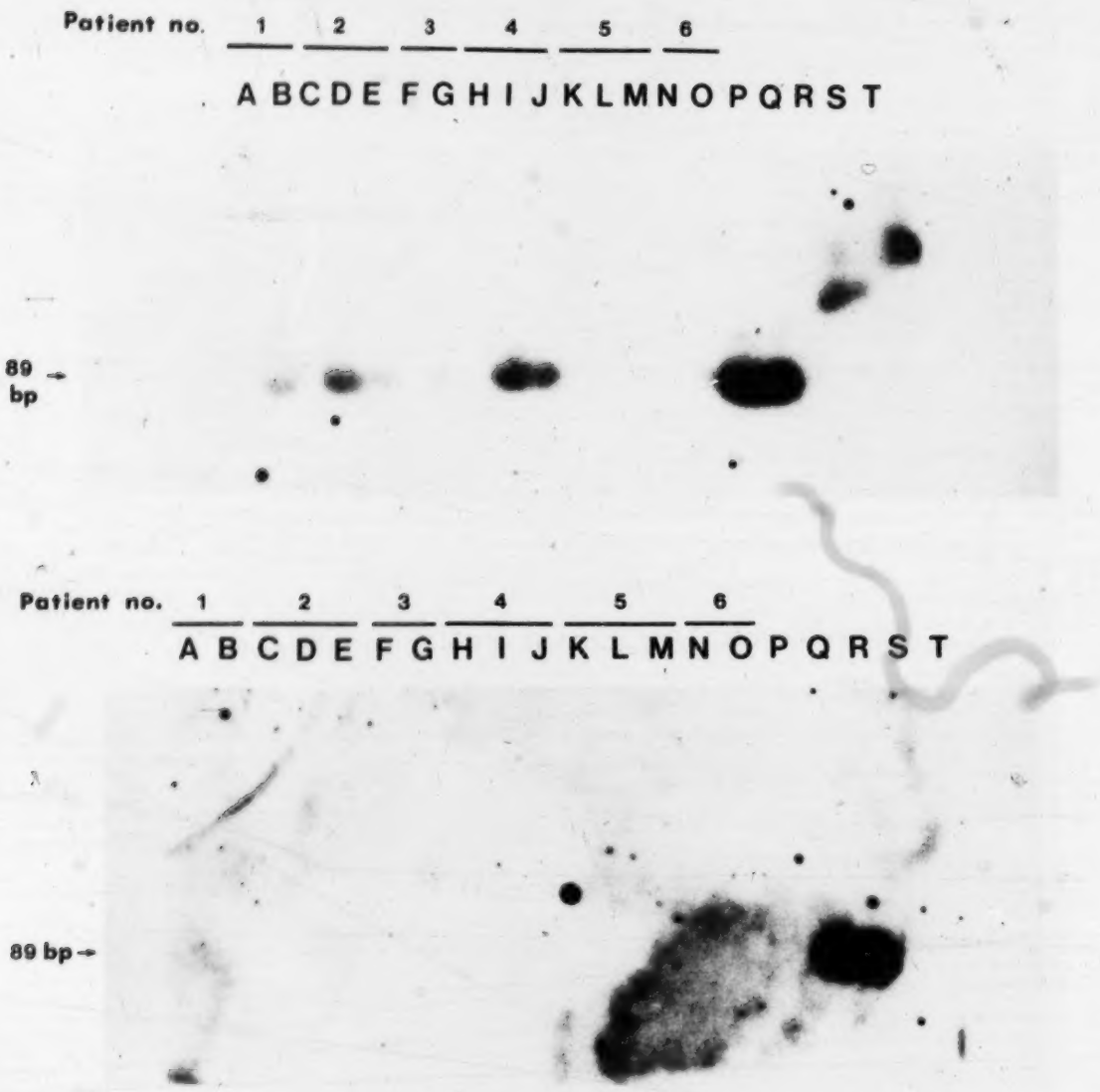

Figure VI-11. Southern analysis of PCR-amplified sequences of the EBNA-2A gene in patients with LPDs. *(Top)* The filter was hybridized with the EBNA-2A-specific (^{32}P)-labeled oligonucleotide probe. Samples *A, C, F, H, K,* and *N* are pre-transplant specimens (all were EBV-negative). Samples *B, D* and *E, G, I* and *J, L* and *M,* and *O* were post-transplant specimens. (Lanes *B, D, E, G, I, J, L,* and O were positive for EBNA-2A. Lanes *P* and *Q* are positive controls and represent the amplification products of the type A variant prototypes B95-8 and Raji, respectively. Lanes *R* and *S* are controls for the type B variants and represent DNAs from the cell lines BL16 and AG876. Lane *T* is a negative control (DNA from cells infected with HSV). All specimens were negative when hybridized with the EBNA-2B-specific oligonucleotide probe. *(Bottom)* The filter was hybridized with the EBNA-2B-specific (^{32}P)-labeled oligonucleotide probe. Only Lanes *R* and *S*, representing the amplified products of the type B variants, were positive.

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CHAPTER VII

SUMMARY AND CONCLUSION

EBV is distinguished from all other human herpes viruses by its ability to immortalize B lymphocytes and by its association with lymphoproliferative disorders and nasopharyngeal carcinoma. EBV has two life cycles: a permissive or productive cycle in epithelial cells, and a latent life cycle in B lymphocytes. The oropharyngeal epithelium is the main site for productive infection. Although the clinical and molecular features of EBV infection have been extensively studied over the past 25 years, a better understanding of the biological effect of EBV reactivation and the clinical effects of a high virus load in human tissues still awaits further study. This project was designed to develop a better method of identifying EBV reactivation states and to quantify EBV reactivation in immunosuppressed patients.

The most common method used to examine the role of EBV in clinical conditions such as lymphoproliferative disorders in immunosuppressed patients and EBV infections in HIV-seropositive individuals has been EBV-specific serology. However, the measurement of antibodies to the virus may not be as accurate as the direct detection of the virus to define reactivation. Furthermore, serology does not provide an estimate of the virus load. Similarly, although it has been used as the "gold standard," lymphocyte transformation has limitations, such as the difficulty in performing the test and its dependency on the transforming phenotype of the EBV variant strains.

The strategy used in this project was to first validate the use of DNA probes for the detection of EBV DNA in oropharyngeal cells by comparing a dot-blot

hybridization technique with the lymphocyte transformation assay. In order to measure the virus load, a quantitative assay was also developed to measure the levels of EBV in oropharyngeal cells. One of the initial problems was to find a DNA probe with a sensitivity and specificity similar to that of the tissue culture system. We found that the *Bam*HI-W fragment, which is a repeat of 3072 bp and is located in the IR1 and spans approximately 20% of the EBV genome, was sensitive and specific in the detection of the virus, providing a relatively simple and rapid method for the longitudinal follow-up of EBV excretion. The dot hybridization helped us describe an association between EBV reactivation and a subset of individuals with new daily persistent headaches. However, further studies are necessary to find the precise role of the virus in headache syndromes.

The *Bam*HI-W probe can be used to detect EBV excretion, but it was not used in quantitative studies since the copy number of this repeating sequence varies from strain to strain. Unique subgenomic fragments were therefore used in the quantitative assay in which the ratio of EBV DNA to human DNA was determined.

Although HIV-seropositive individuals are known to be prone to reactivation of EBV infection, the effects of EBV on the progression of HIV infection are unclear. The prospective analysis of immunologic and viral markers in this population indicated that a high EB virus load was the best single predictor of progression of HIV-induced disease in a subgroup of HIV-seropositive individuals. The actual role that EBV plays in the pathogenesis of HIV infections is not known. Nonetheless, our results suggest that EBV may play a significant role in the natural history of HIV infection.

EBV is known to infect lymphoid tissue, the same target as HIV infections. Co-infection of T or B cells with EBV and HIV may lead to progression of HIV-associated disease through transactivation of HIV by EBV. Other herpes viruses have also been shown to carry genes capable of transactivation of HIV; however, they do not infect the same cell populations as EBV. I believe, therefore, that EBV may be the most important of the viral co-factors in the progression of HIV-associated disease. I am hopeful that our work will be confirmed. Unfortunately, I did not obtain absolute T4-helper cell counts at the time of the original estimation of EBV excretion, but, based on the T4/T8 ratio obtained coincidentally with EBV excretion, I believe high levels of EBV excretion may be a better predictor of progression than absolute T4 levels. If this prediction is true, then it will be important to study the effect of antiviral therapy directed against EBV in slowing the progression to AIDS in HIV-seropositive individuals.

Similarly, the prospective analysis of organ transplant recipients suggested that, after a threshhold of EBV replication is achieved (> 0.2 ng EBV DNA/µg hDNA) in the oropharyngeal epithelium, there is an increased risk of developing LPDs. It is likely that, in the presence of immunosupression and after a certain level of viral replication is reached, the overwhelming infection and subsequent proliferation of B cells results in LPD. To my knowledge, this is the first study to demonstrate that individuals developing LPDs may be identified two to ten weeks before the onset of LPDs, when they are still asymptomatic. The identification of patients at risk of developing LPDs may allow for the early institution of antiviral therapy and the prevention of LPDs after organ transplantation. Furthermore, the PCR analysis and genotypic identification of the EBNA-2 gene, which is responsible for the

transforming phenotype of the EBV variants, suggests that a high virus load of transforming EBV variants (type A) is responsible for the development of LPDs.

This is not a trivial finding, but rather suggests that the expression of viral genes is fundamental to the induction of the disease. The CD23 molecule, a receptor for a B cell growth factor, is upregulated by EBNA-2A. The CD23 molecule or its substrate, a 12 kDa protein (lymphokine) secreted by B cells, may be molecular targets for the design of agents effective in the therapy of LPDs. For instance, the administration of a monoclonal antibody specific for the 12 kDa substrate or a synthetic peptide recognized by CD23 could inhibit the interaction of CD23 and the 12 kDa protein, which in turn could inhibit EBV-induced lymphoproliferation.

EBV may exist intracellularly in episomal or linear forms. Only linear genomes are susceptible to antiviral therapy. To define the episomal or linear nature of the EBV genome, we examined oropharyngeal cells. In order to demonstrate that the stepladder of low molecular weight fragments seen in the Southern blots represents linear forms of the genome, we analyzed Southern blots of DNA extracted from the cell line B95-8 by digesting the DNA with the exonuclease *Bal* 31 to digest the free ends of linear molecules. Unfortunately, I found that this assay lacked reproducibility. A different approach was attempted by treating the cell line B95-8 with acycloguanosine. Acycloguanosine inhibited the low molecular weight bands or linear forms of the EBV genome. Since there is a preponderance of linear forms of the EBV genome in oropharyngeal cells of immunosupressed patients, I predict that the high virus load in these patients may be susceptible to antiviral therapy. This may have very significant implications for the prevention of LPDs in organ transplant recipients and possibly in the

progression to AIDS in HIV-seropositive individuals. My results also suggest that more care may be warranted in the matching of the viral history of donor and recipient in organ transplantation. LPDs occur more frequently in younger recipients. It may be important for seronegative recipients to receive organs from seronegative donors or, at least, from EBV type A-negative donors.

In conclusion, the aim of this study was successfully achieved by validating the use of the DNA probes, which enabled the analysis of the effects of EBV reactivation in the progression of HIV-infection and in the development of LPDs in organ transplant recipients. The results suggest that type A variants are the cause of LPDs in organ transplant recipients. I also defined the threshholds of virus loads in the progression of these EBV-associated diseases. Finally, the analysis of the molecular configuration (linear or episomal) of EBV in the oropharyngeal epithelium and the predictive value of high levels of EBV excretion suggest that early intervention with institution of antiviral therapy may be of benefit. More studies which apply the techniques of molecular virology to understand the pathogenesis and potential benefit of therapy in these conditions is warranted.